EXPERIMENTAL STUDIES ON THE INFECTIBILITY OF *ANOPHELES GAMBIAE* WITH MALARIA PARASITES

by

PATRICIA GRAVES
B.A. (Cantab)

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Faculty of Science
University of London

Ross Institute,
London School of Hygiene
and Tropical Medicine

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ABSTRACT

The feasibility of introducing genes for refractoriness to malaria into populations of *Anopheles gambiae s.s.* is studied.

Morphologically wild-type strains of *A. gambiae s.s.* refractory and susceptible to *Plasmodium yoelii nigeriensis* were selected, although complete refractoriness was not achieved. In crosses between the strains refractoriness showed intermediate dominance.

In a cage replacement experiment, the susceptibility of a population of *A. gambiae s.s.* to *P. y. nigeriensis* was reduced from 100% to 60% by 8 generations of releases of refractory males. The susceptibility of the population showed no reversion towards the original high level after 15 more generations of breeding without releases or artificial selection. This indicated that the low fitness which had been observed in the refractory strains was not conferred by the refractoriness genes, since these were able to recombine into a more well-adapted genome. The population cage results were compared with those expected on a simple theoretical model.

The reliability of a membrane-feeding technique for producing infections of *P. falciparum* in *A. gambiae s.s.* was studied in The Gambia. It was observed that the infectivity of *P. falciparum* gametocyte carriers depended largely on the gametocytaemia. Selection to produce a strain of *A. gambiae s.s.* completely refractory to *P. falciparum* did not succeed, and there were indications that the local strain had a low or zero frequency of genes for refractoriness. It was shown that strains refractory to *P. y. nigeriensis* were not refractory to *P. falciparum*. Oocyst counts of *P. falciparum* were found to be distributed according to the negative binomial distribution.

The possible means of introducing refractoriness genes into mosquito populations are discussed, taking into account the fitness of refractory types both in the presence and absence of malaria infection.

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1. INTRODUCTION

The host-parasite relationship between mosquitoes and malaria is influenced by a variety of factors, which determine the 'infectibility' of a mosquito. These factors include the probability of a mosquito biting a gametocyte carrier, the infectivity of the carrier, the innate susceptibility of the mosquito, the virulence of the parasite and the environmental conditions encountered by the infected mosquito. Certain of the factors, namely the man-biting rate and the probability of daily survival of the mosquito, have long been recognised as being important in the transmission of malaria (Macdonald, 1957). Factors which have perhaps been neglected are the susceptibility of the mosquito to infection and the infectivity of gametocyte carriers.

Problems with insecticide resistance in anopheline mosquitoes have stimulated interest in genetic control methods. Introduction of 'desirable' genes into pest populations, which was reviewed by Curtis (1979), is preferable to eradication, since it does not leave an empty niche to be re-invaded. The central aim of this work is to investigate the feasibility of the reduction of the vectorial capacity of a mosquito population by the introduction of genes causing refractoriness to malaria.

There is an impressive list of mosquito species in which strains have been selected for refractoriness to parasites (see Macdonald, 1967; Wakelin, 1978; Curtis, 1979). However, the majority of these have not involved the human pathogens, but have concerned laboratory model parasite/vector systems. Furthermore, there has been only one example of an attempt to replace a susceptible population with a refractory type in the field, and that concerned an agricultural pest (Foster, 1977). It is important to test the principle of introduction of refractoriness genes, and compare the progress of replacement with that expected on theoretical models. To this end, a laboratory cage replacement experiment concerning introduction of genes for refractoriness to Plasmodium yoelii nigeriensis into a population of Anopheles gambiae sensu stricto is performed. Investigations are also made of the effects which the refractoriness genes, or infection with malaria, have on A. gambiae s.s., because of the influence these effects might have on the prospects for replacement.

The feasibility of population replacement by a laboratory-selected refractory type does not necessarily require that refractoriness be controlled by a single gene. Nevertheless, the genetics of refractoriness are of interest, since the strategy of replacement might be affected depending, for example, on whether refractoriness was dominant or recessive, and the cost would be very much less if refractoriness were dominant. If a single gene were involved, it might be possible to use a more sophisticated method for introduction of the gene into a wild population, by linking it to a mechanism which would 'drive' it in (e.g. hybrid sterility), rather than relying on sheer weight of numbers.

The selection of malaria-refractory strains and study of the inheritance of refractoriness in A. gambiae s.s. are most easily done with rodent malaria, since standardised infection conditions are available. However, since refractoriness to P. falciparum is required, the infectibility of strains of A. gambiae s.s. should be studied with this parasite, for which it is a natural vector. It would be very convenient if strains of A. gambiae s.s. which are refractory to rodent malaria were also refractory to P. falciparum, since at present the only source of infective gametocytes of P. falciparum is from naturally acquired infections. It is essential to establish optimum infection conditions with this parasite before a study of refractoriness to P. falciparum in A. gambiae s.s. can be made.

2. LITERATURE REVIEW

2.1 THE STUDY OF INFECTIBILITY

2.1.1 Susceptibility in the A. gambiae complex

The A. gambiae complex comprises at least six species (Davidson et al. 1967; Davidson & Hunt, 1973) (see Appendix 1A for nomenclature). The sporozoite rate, i.e. the percentage of a sample of mosquitoes dissected which have infected salivary glands, is one measure of the importance of each species in the transmission of malaria. It is clear, though, that the sporozoite rate obtained will depend as much on the host preference, density and longevity of the vector as on its innate susceptibility. Sporozoite rates in A. gambiae s.s. and A. arabiensis have been reported as being approximately the same (Service, 1970; White & Rosen, 1973), or as being much higher in A. gambiae s.s. (White et al. 1972; Highton et al. 1979), depending on the particular conditions. One of the most important factors is the availability of hosts apart from man, since arabiensis is generally more zoophagic than gambiae s.s. Given the differences in man-biting habit and expectation of life, there is no reason to suppose that the two species have different innate susceptibilities to malaria (White et al. 1972), although this possibility cannot be ruled out.

The review by White (1974) summarised the information available from the field on sporozoite rates in the gambiae complex. A. quadriannulatus is strongly zoophagic, and no specimen with sporozoites has been found; this is not to say that it is not susceptible. Species D has been found infected, and is thought to be a vector in its limited locality. Sporozoite rates in A. melas and merus are generally lower than in A. gambiae s.s. (White, 1974; Bryan, 1979). Because of the differences in anthropophily between the members of the A. gambiae complex, susceptibility to human malaria can only be assessed by feeding two or more species on the same gametocyte carrier.

2.1.2 Factors in the vertebrate host influencing infectivity

When studying mosquito susceptibility, it is important that as many factors as possible which influence infectivity of the gametocyte carrier are standardised. This is obviously easiest to do in experimental models. In human malaria, we are restricted to identifying the factors involved and trying to minimise their effects.

Optimal conditions for infecting mosquitoes with rodent or bird malaria have been well investigated. The drawback is that the rodent, bird and mosquito hosts used in the laboratory are seldom the 'natural' ones, and information obtained about mosquito susceptibility may not reflect the 'true' situation, since the growth pattern and behaviour of the parasites may be altered (Yoeli et al. 1966). Nevertheless, experimental models have provided a great deal of information about infectivity of gametocytes, susceptibility of mosquitoes and the sporogonic cycle in general.

Since Yoeli and Wall (1952) first performed the cyclical transmission of P. berghei, numerous authors have shown that the gametocytes of rodent malaria are most infective during the early stage of an infection (e.g. Vincke & Bafort, 1968; Wéry, 1968; Bafort, 1971). Build-up of immunity may be responsible for the loss of gametocyte infectivity during the course of an infection, as has been well discussed by Mendis (1980). Obtaining high, repeatable infections with P. berghei or yoelii also depends on using a parasite which has recently undergone cyclical transmission through mosquitoes (Wéry, 1968).

The Aedes aegypti/P. gallinaceum system has also proved useful for studying factors affecting infectivity of gametocytes, and mosquito susceptibility. Eyles (1951) showed a clear linear relationship between gametocytaemia and mean oocyst number, by diluting or concentrating gametocytes from one bird to known numbers and then feeding them to mosquitoes through membranes. In this elegant and successful experiment he was able to eliminate factors affecting infectivity varying between different birds. Data from direct feeds on different birds showed a significant regression of mean oocyst number on gametocytaemia, although the graph suggests that a curvi-

linear relationship may apply. It was also shown that the number of oocysts per gut in a given batch of mosquitoes was related to the percentage infected by a curve of hyperbolic form which approaches 100% infected, suggesting that few or none of the mosquitoes in the strain of Ae. aegypti used were refractory to P. gallinaceum.

It has long been known that, as for rodent malaria, the highest oocyst counts of P. gallinaceum are obtained in feeds taken before the peak of parasitaemia, and there is evidence that this is due to a build-up of immunity as the infection progresses (Eyles, 1952a,b; Huff et al. 1958). There is no doubt that transmission-blocking immunity can be produced artificially in P. gallinaceum (Carter & Chen, 1976; Gwadz, 1976), as well as in P.y. nigeriensis (Mendis & Targett, 1979).

Much of the work with human malaria has been concerned with assessing natural infectivity for epidemiological studies, although some studies have been done on patients undergoing malaria therapy. Considering first the effect of gametocyte number on infectivity, it is to be expected that there is a threshold number for infection. This is extremely low; it is of the order of 1-10/mm^3 for P. falciparum (Kligler & Mer, 1937; Young et al. 1948; Muirhead-Thomson & Mercier, 1952; Muirhead-Thomson, 1954). In general, the proportion of a batch of mosquitoes infected with P. falciparum, and the intensity of infection within a batch, are proportional to the gametocytaemia (Draper, 1953; Jeffery & Eyles, 1955; Bray & Burgess, 1964; Rutledge et al, 1969). However, as Rutledge et al. (1969) pointed out, the relationship is non-linear, since beyond a certain gametocytaemia the percentage infected has reached 100% and can increase no further. Burgess (1960) and Wilkinson et al (1972) found no correlation between gametocytaemia and infectivity to mosquitoes. Many authors also noted the surprising fact that people with very high gametocyte densities occasionally fail to infect mosquitoes (James, 1931; Young et al. 1948; Muirhead-Thomson & Mercier, 1952; Muirhead-Thomson, 1954).

There is some evidence that the stage of infection influences the infectivity of P. falciparum (Jeffery & Eyles, 1955; Ward et al. 1972). Gametocytes of P. falciparum take several days to mature

(Smalley, 1976), and gametocytes may build up to over 1,000/mm^3 in this non-infective period (Jeffery & Eyles, 1955). In the later period of infection, however, the evidence is that antibodies to gametocytes or gametes (if present) have little effect on gametocyte infectivity (Smalley & Sinden, 1977; Carter et al. 1980).

It is hard to generalise about the effect of age on infectivity of gametocyte carriers, since gametocyte rates in different age-groups vary in different areas according to the level of malaria transmission. Carter et al (1980) concluded, from data of Muirhead-Thomson (1954, 1957), that all age groups contribute to the infective reservoir according to their level of gametocytaemia. The observation of Muirhead-Thomson (1954) that infants under the age of one year were poor infectors despite having high gametocytaemias remains unexplained.

In infectivity studies involving naturally-acquired human malaria (e.g. Young et al. 1948), use of antimalarial drugs may have affected the results. Chloroquine has no effect on the growth or infectivity of mature P. falciparum gametocytes, although it kills immature gametocytes (Jeffery et al. 1956; Smalley, 1977). Chloroquine given to patients with naturally acquired chloroquine-resistant malaria was found to increase the median oocyst values in A. balabacensis (Wilkinson et al. 1976). Primaquine and proguanil, on the other hand, caused disappearance of gametocytes from the circulation, whilst pyrimethamine caused loss of infectivity without removing the gametocytes (Shute & Maryon, 1954; Jeffery et al. 1956; Rieckmann et al. 1969). It seems therefore that treatment with pyrimethamine before an infective feed may be responsible for a small proportion of the failures of high gametocyte carriers to infect. Other anti-malarial drugs cannot be implicated in this way.

The theory of Hawking et al (1971) that there is a circadian rhythm of infectivity of P. falciparum gametocytes, corresponding to the time of peak mosquito biting activity, has been disproved by Bray et al (1976). Once gametocytes are mature, their infectivity persists for up to 20 days (Jeffery et al. 1956; Smalley & Sinden, 1977).

2.1.3 Environmental factors affecting infectibility

The most important factor affecting whether sporogony can proceed in mosquitoes which have fed on an infective gametocyte carrier is temperature. The range of permissive temperatures for development of P. falciparum is 19 to 32°C, whilst P. vivax has a lower minimum of 15°C (Macdonald, 1957). The length of the sporogonic cycle is dependent on temperature.

Rodent malaria seems to be more strictly temperature controlled than human. The successful routine cyclical transmission of P. berghei depended on the discovery that low temperature was necessary for sporogony (Yoeli et al. 1964). The sensitive period for high temperature is early in the sporogonic cycle (Vanderberg & Yoeli, 1966). The optimum temperature for development of P.y. nigeriensis was found to be 24°C (Killick-Kendrick, 1973).

There are numerous reports of viruses or virus-like particles in mosquitoes infected with malaria parasites, either solely within the parasite or also present in the midgut wall (e.g. Davies et al. 1971; Bird et al. 1972). However, controlled studies concerning the effect which these viruses have on the growth of oocysts on the midgut are few. Bertram et al (1964) showed that in Ae. aegypti. numbers of oocysts of P. gallinaceum were suppressed if the mosquitoes were infected with Semliki Forest virus as well as the malaria. The stress of the double infection caused high mosquito mortality which would interrupt transmission. Infections with microsporidian parasites were also found to reduce numbers of oocysts and sporozoites in malaria-infected mosquitoes, as well as cause high mosquito mortality (Hulls, 1971; Savage et al. 1971; Ward & Savage, 1972).

Reduction in the microbial flora on the gut of Culex quinquefasciatus (see Appendix 1B for nomenclature of C. pipiens group) by feeding of antibiotics increased its susceptibility to P. relictum (Micks & Ferguson, 1961). Reduction in competition for nutrients by changes in bacterial flora may also be responsible for the influence of acids, bases, salts, antibiotics, hormones and vitamins in the diet of Ae. aegypti. which stimulated or depressed susceptibility depending on the substance and sometimes on its concentration (Terzian

et al. 1953; Terzian & Stahler, 1960).

Other factors which may affect the infection rate in mosquitoes are the size of the bloodmeal and the size and age of the mosquitoes. Whilst there is some indication that the quantity of blood ingested is of importance (Hovanitz, 1947), above a certain minimum amount it plays a minor role compared to other factors. Ward (1963) found no relationship between the size of adult Ae. aegypti and oocyst number of P. gallinaceum. The age of mosquitoes does, however, affect their susceptibility. Older mosquitoes (2-4 weeks old) were less susceptible to P. gallinaceum than younger ones, although full susceptibility could be restored to the older group by an uninfected bloodmeal given within nine days prior to the infected bloodmeal (Terzian et al. 1956).

2.1.4 Genetic factors affecting infectibility

Genetic factors in mosquitoes affecting susceptibility to vertebrate pathogens have been noted in a large number of cases involving filariae, viruses and malaria. In Ae. aegypti, the sex-linked recessive gene f^{m} controls susceptibility to Wuchereria bancrofti and two species of Brugia (Macdonald, 1962; Macdonald & Ramachandran, 1965), whilst its allele f^{m2} controls susceptibility to Waltonella flexicauda (Terwedow & Craig, 1977). A different locus, ft, controls susceptibility to species of Dirofilaria (Zielke, 1973; McGreevy et al. 1974). In the case of C. quinquefasciatus, selection for refractoriness to W. bancrofti on a Liberian strain of mosquito was successful (Zielke & Kuhlow, 1977), although these authors found in common with others (Singh & Curtis, 1974) that selection for refractoriness on the more susceptible Indian C. quinquefasciatus was not successful. In mosquitoes and midges, strains refractory and susceptible to viruses have been selected (Jones & Foster, 1974; Hardy et al. 1978).

There is no doubt that genetic factors influence mosquito susceptibility to malaria. In the past, an artificial distinction seems to have been created between the concepts of 'natural immunity' of a mosquito species to a particular malaria parasite, and of artificially (i.e. laboratory) selected refractoriness. Natural immunity, which is encompassed in the term 'species specificity' is

often illustrated by the case of the culicine mosquitoes, which do not transmit human malaria. The very term 'species specificity' creates the impression that there are very clear-cut distinctions between mosquito-parasite combinations which work, and those which do not.

That 'species specificity' is a rather misleading term is well illustrated by the rodent malaria, P. berghei, in which we find a complete spectrum ranging from complete insusceptibility to well-adapted susceptibility in different mosquitoes (Yoeli, 1973). In C. salinarius, refractoriness is complete and even exflagellation and ookinete formation are prevented. Ookinetes do form in Ae. aegypti, but cannot penetrate the midgut; oocysts and sporozoites form to an increasing extent in A. quadrimaculatus, A. aztecus and A. stephensi. In A. dureni, the natural vector, large numbers of oocysts and infective sporozoites are formed. P. gallinaceum provides a further example: C. pipiens (see Appendix 1B) is generally regarded as being non-susceptible to this species, whilst Ae. aegypti is very susceptible (Huff, 1927; Weathersby, 1952). However, closer studies showed that in fact some oocysts are formed in C. pipiens (Noblet & Weathersby, 1973), and a large proportion of the parasites degenerate in the susceptible species (Huff, 1934).

There are numerous examples of species of mosquito varying quantitatively in their susceptibility to particular malarias (e.g. Wilkinson et al. 1972; Collins et al. 1975; Collins et al. 1976a). Not surprisingly, many authors reporting species differences in susceptibility also found within-species differences (Boyd, 1941; Rutledge et al. 1970; Collins et al. 1976b). Mosquito species and strain susceptibility cannot be considered in isolation, however, since it is affected by the infectivity of the parasite. Again, there are numerous reports of strain differences in malaria in this respect (Huff, 1940; Jeffery et al. 1954; Collins et al. 1963; Bennett et al. 1966; Collins et al. 1976a,b).

Within-species variation in susceptibility to P. vivax and two strains of P. falciparum was demonstrated in A. albimanus (Warren et al. 1977) and in A. freeborni (Collins et al. 1979). Strains differing in pupal colouration alleles at two loci were selected from within one strain of A. albimanus from El Salvador (Warren et

al, 1975). Significant differences in the malaria susceptibility of these strains was found. It is not clear whether the differences in susceptibility were due to pleiotropic effects of the colour alleles, or to chance linkage between the pupal colour alleles and alleles affecting susceptibility. However, when the same pupal colour phenotypes were picked out from mixed strains of A. albimanus on another occasion, differences in susceptibility were found once more but the associations of particular pupal colours with high or low susceptibility were not the same as in the first experiments (Warren et al. 1979). This strongly suggests that the linkage explanation is the correct one.

There is no reason to suppose that different mechanisms are operating in refractory strains selected in the laboratory to block parasite development than are operating to produce the phenomenon of species variation in susceptibility in the wild. The natural situation can be assumed to have arisen from a process of natural selection and adaptation of the parasite to the host. This process can be clearly seen to have occurred in situations where adaptation of particular strains of malaria to their local populations of mosquito is found. A. quadrimaculatus from Florida was less susceptible to a strain of P. falciparum from Panama, than were two strains of A. albimanus, one from Panama and one from the Florida Keys (Jeffery et al. 1950). However, A. quadrimaculatus was more susceptible to P. falciparum from South Carolina than was the Panamanian A. albimanus (Eyles & Young, 1950). The European mosquitoes A. atroparvus and A. labranchiae, which were good vectors of European P. falciparum, were shown to be almost completely refractory to Kenyan P. falciparum (Ramsdale & Coluzzi, 1975).

Since species specificity is such a variable phenomenon, it is not surprising that susceptibility to malaria can be influenced by selection in the laboratory. Partial susceptibility within a species was described by Huff (1927) as 'individual immunity'. In a survey of the susceptibility of many species of mosquito to three bird malarias, he noted when assessing the species specificity to P. cathomerium that even in 'susceptible' mosquitoes, certain individuals did not become infected even when they must have received thousands of gametocytes in the bloodmeal. Other mosquitoes in the

same batches became highly infected. Whilst not discounting factors such as bacterial infections affecting susceptibility, he suggested that such individuals were inherently immune, and remained so throughout their life. He later showed that when individual C. pipiens were given two bloodmeals on the same species of malaria at five day or longer intervals, they were either susceptible or insusceptible at both, with only two out of 60 exceptions (Huff, 1930).

Huff (1929) showed by selection of single families descended from infected or uninfected C. pipiens that 'individual immunity' to P. cathemerium was hereditary. The susceptibility in one line was reduced to 7.4% after ten generations, whilst the highest susceptibility achieved was 67.8% compared with the control strain which was 28.1% susceptible. Selection of refractory and susceptible lines was later repeated with C. pipiens and extended to C. quinquefasciatus which also showed good response to selection (Huff, 1931). Study of the inheritance of susceptibility presents some problems, since only females can be tested for susceptibility. After five generations of single family selection on C. pipiens, 42 uninfected female offspring were obtained from a single mother infected with P. cathemerium, and this is given as evidence that refractoriness is dominant (Huff, 1931). However, since there were no controls, the possibility of a failed infection cannot be ruled out. Huff also states that the numbers of uninfected and infected offspring in following generations fit in with predictions of ratios based on recessive susceptibility controlled by a single gene, but in fact the numbers are small and consistent with other hypotheses.

After Huff's pioneering work, several authors attempted to select strains of mosquito with increased or decreased susceptibility to malaria. Hovanitz (1947) failed to alter the susceptibility of Ae. aegypti to P. gallinaceum after six generations of selection, and there was no parent-offspring correlation in oocyst number in the selected lines. Lack of such correlation would indicate that there is no heritable variation in susceptibility to P. gallinaceum, but in fact such variability was later found (Ward, 1963; Kilama & Craig, 1969). Thus it seems that in Hovanitz's work, environmental variation in the infections was obscuring any genetic variation; variable infectivity of gametocyte carriers may have been a factor

since it was impossible to feed parents and offspring on the same carrier. Boyd and Russell (1943) were unable to select refractory and susceptible strains of A. quadrimaculatus to P. vivax, probably for the same reasons of excessive variability in infection conditions.

Other authors were reasonably successful. After six generations of selection, Trager (1942) produced a strain of Ae. aegypti with increased susceptibility to P. lophurae. Its average susceptibility was 62% compared to 30% in the control, and this difference was maintained over a year. Micks (1949) increased the susceptibility of C. pipiens to P. elongatum from 13 to 49% by six generations of selection. The Rockefeller Foundation (1948,1950) crossed selected lines of A. quadrimaculatus differing in their susceptibility to P. gallinaceum and the F_1 was intermediate in susceptibility.

Ward (1963) described the selection over 26 generations of a strain refractory to P. gallinaceum (the 'Low line') from a control Malayan strain of Ae. aegypti which was 96% susceptible on average. Attempts to select a 'High line' (i.e. with increased mean oocyst count) from the same stock failed. After 14 generations of selection for refractoriness a subline 'AB' was established from the Low line and no further selection was practised on it. Susceptibility in the AB line had not reverted to the control level 13 generations after cessation of selection.

In reciprocal crosses between the AB line and the Malaya control, F_1 females were found to have mean oocyst counts which were intermediate between those of the parental types. It is also stated that when the F_1 offspring of the cross 'Low line X Malaya' were backcrossed to the Low line, there was a tendency to bimodality in the distributions of oocyst counts obtained in the backcross progeny. However, the graphs of these distributions for the two reciprocal backcrosses are not convincingly more bimodal than the corresponding graph given for the F_2 generation. Nevertheless, Ward proposed that susceptibility is under the control of a single Mendelian factor or group of closely linked genes, exhibiting incomplete dominance. In this model, the genotype S_1/S_1 denotes low or no susceptibility, and S_1/S_2 and S_2/S_2 are the genotypes for intermediate and high susceptibility respectively. The latter two genotypes are phenotypically virtually indistinguishable

due to overlap between the oocyst distributions. This is probably the reason that selection for a strain of high susceptibility failed, since individuals selected for the High line were likely to be heterozygotes.

There is also some overlap between the oocyst count distributions for the low and intermediate phenotypes (genotypes S_1/S_1 and S_1/S_2, according to the model), which may explain why it took 26 generations to reach an almost completely refractory line. To avoid inbreeding depression, individuals which had low oocyst counts were selected rather than those which were completely refractory.

Ward (1966) used Huff's technique of double infective feeds to demonstrate that Ae. aegypti which were susceptible or refractory to an initial infective feed responded in the same manner when given a second infective feed three days later. Oocysts from the second feed could be distinguished by their smaller size. With only one exception, all mosquitoes infected during the first meal also developed oocysts after the second meal, whilst those at first refractory remained so.

The rank correlation coefficient for oocyst numbers between two successive infective feeds for individual mosquitoes of the three lines was found to be lowest in the Malaya line, intermediate in the AB line and highest in the Low line. This is interpreted by Ward as indicative of a quantitative character controlled by several factors. He suggests that the Low line is genetically more homogeneous, having undergone more generations of selection than the other two lines, and therefore would be expected to exhibit a higher degree of correlation in a quantitative character. This interpretation seems to be fallacious, however, for two reasons. Firstly, since in the Low line the majority of oocyst counts are zero, the amount of variation possible is lower than in the other two lines, and the rank correlation coefficient will therefore be higher due to a 'scale effect'. Secondly, the correlation coefficient has been calculated between different feeds in the same individual, and the correlation within individuals is expected to be the same for each strain regardless of the degree of homozygosity in the strain. Greater genetic variation in an unselected line would only be apparent between individuals.

Kilama and Craig (1969) also studied susceptibility to P. gallinaceum in Ae. aegypti, although with different results to those of Ward (1963, 1966). Kilama and Craig (1969) established two strains of Ae. aegypti refractory to P. gallinaceum within one generation of selection (although one of the strains was almost completely refractory before selection was started). There was a clear distinction between refractory and susceptible individuals. Refractory mosquitoes usually had no oocysts and never more than 6, whilst susceptible mosquitoes had an average of above 50 oocysts. 10 oocysts was taken as the point of demarcation between the two classes. The refractoriness factors in the two refractory strains were shown to be allelic by a complementation test.

Crosses to a susceptible strain established that refractoriness is controlled by a single autosomal recessive factor, 'plasmodium susceptibility' (pls). Linkage studies showed that pls is located on linkage group 2 between the markers Silver-mesonotum (Si) and Dieldrin resistance (Dl). The distances between pls and Si and between pls and Dl are 8 and 17 crossover units respectively. This work provides the first convincing example of monofactorial inheritance of refractoriness to malaria in a mosquito species.

The C. quinquefasciatus/P. cathemerium system, mentioned previously by Huff (1931) was also studied by Dennhöfer (1971). He was able to produce pure-breeding refractory and susceptible lines after eight generations of single family selection from a single female, which must have been carrying refractory and susceptible alleles. The presence of sporozoites in the salivary glands was used as the criterion of susceptibility.

Dennhöfer was able to infer the mode of inheritance from the study of the 'family-tree' which gave rise to the pure-breeding lines. In several cases in the F_4 generation, refractory mothers gave either all or some susceptible offspring; when such segregation did occur, refractory and susceptible individuals were in a 1:1 ratio. Susceptible females either had all susceptible offspring (12 cases) or had offspring which were susceptible and refractory in a 3:1 ratio when the numbers were pooled (27 cases). Never did it happen that susceptible mothers gave only refractory offspring. Reciprocal crosses between refractory and susceptible strains also gave evidence

that susceptibility is controlled by a single gene which is dominant (although not completely so; F_1 mosquitoes had reduced numbers of sporozoites in the glands). Huff (1931) had suggested that susceptibility to P. cathemerium in C. pipiens was recessive. The gene concerned was mapped to the third linkage group by the use of the recessive marker ruby eye on linkage group 2, which was present in the original strain from which the lines were set up.

Corradetti et al (1970) selected strains of A. stephensi which were refractory and susceptible to P. gallinaceum. The results of Frizzi et al (1975) suggest that susceptibility and refractoriness of A. stephensi to P. gallinaceum are controlled by a single gene, the refractoriness being dominant. Reciprocal crosses between line A (susceptible, with an average of 30 oocysts per midgut) and line B (refractory, with 3% of females developing oocysts) gave 169 refractory and 14 susceptible phenotypes. This is interpreted as showing that refractoriness is dominant, but that other variables such as 'male genotype' or 'modifiers' are acting. A more probable explanation is that line B was not homozygous for refractoriness. If the 3% of susceptibles are homozygotes for a susceptibility gene, then the frequency of heterozygotes would be 0.28 by the Hardy-Weinberg Law. It is therefore quite likely that selected individuals in the B line, or B parents in a cross, were heterozygotes. Selection of a pure refractory line when refractoriness is dominant must be done by single family selection. It is not possible for Frizzi et al (1975) to distinguish between incomplete dominance of refractoriness, or complete dominance disguised by an impure line B.

Results of some of the other crosses do suggest a single dominant gene mechanism controlling refractoriness, although one backcross of F_1 X susceptible parent gave offspring differing significantly from a 1:1 ratio, but with an excess of refractory mosquitoes. This cannot be explained by postulating hidden susceptible alleles in the refractory line. Further information about the criteria used to distinguish refractory and susceptible individuals, and the methods used to select the lines, is needed for full assessment of the results of Frizzi et al (1975).

Van der Kaay and Boorsma (1975) reported the selection of A. atroparvus for susceptibility and refractoriness to P. berghei. After 14

generations of single family selection, the susceptible line was on average 98.7% positive for infection with a mean oocyst number of 189. The refractory strain had a susceptibility of 6.9% by generation 14 and a mean oocyst number of 5. A control strain showed some fluctuation in susceptibility over the period of the selection but no significant trend in either direction.

The susceptible (S) and refractory (R) strains were maintained for a further six generations, during most of which selection was carried out (van der Kaay & Boorsma, 1977). By F_{18}, 100% susceptibility had been reached in the S line, and by F_{20} the oocyst number had also shown a marked increase to an average of 456 oocysts per mosquito. A completely refractory line was not achieved, although by F_{20} the susceptibility had shown a slight overall decrease since F_{14} to 5% and the mean oocyst number was down to 1 per mosquito. Sporozoites in the S strain were found to be infective to mice up to 59 days after an infective bloodmeal.

Reciprocal crosses between the S and R strains gave F_1 mosquitoes showing intermediate susceptibility (averaging 50%). The mean oocyst number was strikingly similar (31 and 32 respectively) in the 2 reciprocal crosses. Backcrosses to the R strain gave offspring showing 11% susceptibility, whilst backcrosses to the S strain gave offspring with 66% susceptibility. Whilst van der Kaay and Boorsma (1977) state that this is indicative of multiple interacting genes controlling susceptibility to an infection with P. berghei, in fact this data is not inconsistent with a hypothesis of a single factor controlling susceptibility which shows incomplete dominance. In a backcross of the F_1 to the susceptible parent, we would expect RS and SS offspring in an equal ratio. If the RS are 50% susceptible and the SS 100%, on balance we expect 75% susceptibility. The value obtained in this cross was 66%. By a similar argument, in a backcross of F_1 to the refractory parent we expect an overall susceptibility of 25%, whilst a value of 11% was actually obtained. Further data on whether the backcross progeny showed a uni-modal or bi-modal distribution of numbers of oocysts are needed to clarify the situation.

Al-Mashhadani et al (1980) studied the genetics of susceptibility of A. gambiae to P. berghei. Selection for a highly susceptible line was started from a strain of A. gambiae s.s. with 50-70% susceptibility.

The rate of infection increased from 52.4% to 95.7% in one generation, and single family selection for five more generations resulted in a strain with 100% susceptibility, termed 'PB'. Selection for refractoriness was carried out on a different strain which was carrying the recessive sex-linked gene white-eye, and which had susceptibility to P. berghei fluctuating between 30-63%. The rate of infection in the selected line 'LD' decreased from 62.5% to 27.3% in one generation, with further decreases until the eighth generation when susceptibility was 0% for sporozoites, whilst 5% of the mosquitoes dissected had a few degenerate oocysts. Three further generations of selection on LD brought its susceptibility down to 0% (Al-Mashhadani & Davidson, 1976).

In the work of Al-Mashhadani et al (1980), control infections of A. stephensi 'BEECH' (100% susceptible) were always carried out to check the infectivity of the parasite. Susceptibility was assessed by dissection of both stomachs and salivary glands, and intensity of infection was assessed by the 'sporozoite index', an estimate of the sporozoite density in positive mosquitoes. The success of the selections in reaching full refractoriness and susceptibility in the two lines was probably due to the fairly stringent selection for susceptibility/refractoriness, and to the ability to carry out nine generations of full sib-mating.

Crosses between the two lines LD and PB showed that susceptibility was incompletely dominant to refractoriness. However, the progeny of the reciprocal crosses differed in their susceptibility. Crosses with LD as the female parent consistently gave F_1 progeny with significantly higher susceptibility (averaging 93.6%) than the reciprocal cross (averaging 63.5%). A similar difference between the F_1s was apparent in the 'sporozoite index'. The direction of the difference between the two crosses was such that it cannot be explained by maternally inherited cytoplasmic factors, since the male parent seemed to exert greater effect on the susceptibility of the F_1.

The backcrosses to LD gave, as expected, progeny with lower susceptibility than the F_1, but backcrosses to PB had no higher susceptibility than the F_1. The proportions of susceptible offspring from the different backcrosses and the F_2 are not consistent with a single-

gene model (either autosomal or sex-linked) or an additive polygenic model for the control of refractoriness/susceptibility. However, a significant correlation was found between white-eye (for which LD is homozygous) and refractoriness to P. berghei in those backcrosses and the F_2 in which white-eye was segregating. This indicates either that the white-eye gene has a pleiotropic effect on refractoriness, or that a gene linked to it on the X-chromosome has an influence on refractoriness. However, this factor must be one of a number of interacting polygenes, since additive polygenic inheritance cannot explain the difference in susceptibility between the two reciprocal F_1s. Furthermore, since the stock from which LD was derived was homozygous for white-eye and yet was about 60% susceptible, a pleiotropic effect of white-eye cannot be the complete explanation for the refractoriness of LD.

2.2 THE SPOROGONIC CYCLE

2.2.1 The normal course of sporogonic development

Once strains of mosquito have been established which are refractory to malaria investigation of the cause of refractoriness is of considerable interest. For this purpose knowledge of the normal development of malaria in the mosquito is needed. The process of mosquito infection in rodent malaria has recently been extensively reviewed by Sinden (1978).

Ookinetes are formed in the blood in the gut of the mosquito after fusion of the male and female gametes. Rodent malaria ookinetes are crescent-shaped, with the anterior end blunter than the other. The electron microscope has shown that the ookinete is bound by a pellicle and two membranes beneath which are a layer of microtubules (Garnham et al. 1969; Canning & Sinden, 1973; Davies, 1974). A prominent feature of the ookinete of P. y. nigeriensis is the 'apical complex' comprising a polar ring, a collar continuous with the inner pellicle membrane, an electron-dense ring to which microtubules are attached, and rhopteries (Canning & Sinden, 1973). Although Davies (1974) did not observe the electron-dense ring, dense bodies probably equivalent to rhopt ries were seen. The apical complex may be involved in penetration of the stomach wall.

Ookinetes of P. berghei were observed to move by annular waves of contraction, or by forward gliding combined with rotation around the longitudinal axis (Freyvogel, 1966; Speer et al. 1975). Ookinetes isolated by Weiss & Vanderberg (1976) moved with the blunt end anterior, or alternatively pivoted round the posterior end which was attached to a slide. Curving of the body could be brought about by contraction of the longitudinal sub-pellicular microtubules, whilst contraction of microtubules in the 'snout' containing the polar ring could result in circular or boring movements (Garnham et al. 1969; Davies, 1974).

Ookinetes of P. berghei were observed penetrating the gut wall of A. stephensi 16-19 hours after a bloodmeal (Freyvogel, 1966), whilst those of P. gallinaceum passed through the gut wall of Ae. aegypti from 15-30 hours after a bloodmeal (Stohler, 1957). By cine-photography of peritrophic membranes of Ae. aegypti carefully removed from around a coagulating bloodmeal, Freyvogel (1966) concluded that ookinetes of P. gallinaceum are temporarily held up by the peritrophic membrane. They then actively work their way through with the aid of annular waves of contraction. Once through, their posterior end remains fixed to the membrane while they perform 'searching movements' until the anterior end contacts the brush border of the epithelium. Although some authors observed the ookinetes lying parallel to the gut wall before penetration (Huff, 1934; Stohler, 1957), the general view is that the ookinetes migrate with the apical complex directed towards the basal lamina, and that migration is intracellular (Sinden, 1978). The structure of the ookinete commences breaking down after it has migrated to the basal lamina.

Detailed descriptions of oocyst formation have been published for rodent malaria (Sinden, 1978) and P. falciparum (Sinden & Strong, 1978). Within the developing oocyst, the sporozoites develop by budding off from a central mass, the 'sporoblastoid body', in which numerous nuclear divisions have taken place (Vanderberg et al. 1967; Howells & Davies, 1971; Schrevel et al. 1977). The enlarging oocyst is covered by the basal lamina of the gut, and later also by a fibrous capsule which is probably of parasite origin (Sinden, 1978). The capsule may have supportive or protective functions. Growth of the oocysts was observed to stretch and rupture the basal lamina,

exposing the fibrous wall structure in P. y. nigeriensis (Sinden, 1975), but in P. falciparum 9-10 day oocysts were still usually covered by a continuous layer (Sinden & Strong, 1978). Breakdown of the oocyst wall and basal membrane may be essential for sporozoites to be able to escape, although there is some evidence that they may exit through small pores (Sinden, 1974). Once in the haemocoel, the sporozoites are exposed to the cellular and humoral defence reactions of the mosquito.

2.2.2 Development of parasites in refractory mosquitoes

Parasites in refractory mosquitoes may be destroyed whilst still in the bloodmeal; they may fail to penetrate the stomach wall, or they may be destroyed after reaching the haemocoel. It is not to be expected that the mechanism controlling refractoriness would be the same in each mosquito/parasite system. Some studies have involved comparisons between refractory and susceptible strains of one mosquito species, whilst others have used susceptible and 'refractory' species of mosquito.

It is known that the digestive enzymes of Ae. aegypti cause damage to P. gallinaceum ookinetes in vitro (Gass, 1979) and suppress development of oocysts in vivo (Gass, 1977). However, Huff (1927) concluded that digestion proceeded at the same rate in both susceptible C. pipiens and refractory Ae. sollicitans, and that ookinetes of P. cathemerium formed to the same extent in both cases. In later work using the double feeding technique to identify refractory individuals in C. pipiens (Huff, 1934), he showed that all the ookinetes formed in refractory individuals later degenerated. A large proportion of the parasites also degenerated in the susceptible individuals. Hilton (1974) observed normal and degenerating ookinetes of P. y. nigeriensis in several strains of A. gambiae s.s., but states that in one strain which had been shown not to develop any oocysts, the majority of the ookinetes had degenerated by 14-16 hours after the bloodmeal.

The work of Stohler (1957) and Freyvogel (1966) suggested that the peritrophic membrane in the gut wall might form a barrier to ookinetes. It possibly acts as a barrier to other parasites, e.g. viruses, although this is not certain (Stohler, 1961; Orihel,

1975; Houk et al, 1979). The time at which a peritrophic membrane (P.M.) is formed after a bloodmeal is species specific. In Ae. aegypti, a P.M. is visible in light microscope sections from about five to eight hours until 48 hours after the bloodmeal (Stohler, 1957), whilst in the electron microscope a P.M. is visible within half an hour of the bloodmeal (Bertram & Bird, 1961). Its layered structure is formed from material secreted by the epithelium, and it is initially liquid and viscous but gradually hardens until at 30 hours it is tough and brittle and too hard for ookinetes to pass (Stohler, 1957).

In A. gambiae s.l., a P.M. is apparent in light-microscope sections about 13 hours after the bloodmeal, becoming thickest at 30 hours, whilst in A. stephensi it is formed much later and is not apparent until 32 hours (Freyvogel & Stäubli, 1965). The same authors state that a P.M. is not formed as such in A. atroparvus. However, van der Kaay et al (1978) found that in a strain refractory to P. berghei. ookinetes were not able to penetrate a layer adjacent to the gut epithelium which may be its equivalent.

Thus there seems to be a 'race against time' for ookinetes to find the stomach wall and penetrate the P.M. before they either succumb to digestive enzymes or the P.M. becomes too hard and dense to penetrate. It is possible that refractory mosquitoes either have increased secretion of digestive enzymes, a faster hardening of the P.M. or alteration of recognition sites on the P.M.

The haemolymph may also be involved in refractoriness. When Kilama (1972) studied the fate of P. gallinaceum in refractory and susceptible strains of Ae. aegypti. he observed in midgut sections that parasite development was similar in the two strains until 50 hours after the bloodmeal. Young oocysts formed in both strains, but those in the refractory strains then degenerated and were not visible after 60 hours. A haemocoelic factor may be involved in their degeneration. The haemocoel is known to contain various haemocytes which are involved in defence reactions e.g. in the encapsulation of filariae (Esslinger, 1962). However, it is difficult to see how they could recognise oocysts, which are covered with host tissue in the form of the basement membrane. A non-cellular immunity may be involved,

or the haemolymph in refractory mosquitoes may simply lack nutrients required by the developing oocysts.

A systemic haemolymph factor in refractory C. pipiens was found to prevent the further development of gametocytes, oocysts or sporozoites of P. gallinaceum or P. fallax when they were injected directly into the haemocoel, whilst in susceptible Ae. aegypti or Ae. albopictus, ectopic oocysts formed and some salivary gland infections were obtained (Weathersby, 1952; 1954; 1960; Weathersby & McCall, 1968). It was possible to transfer the 'immunity' from C. pipiens to Ae. aegypti by connecting the haemocoel of two mosquitoes by parabiotic twinning (Weathersby, 1963; 1965). Reduction in susceptibility was also observed when Ae. aegypti were fed on crude extracts of C. pipiens (and, to a lesser extent, on extracts of Ae. aegypti itself), confirming the systemic nature of the refractoriness factor (Weathersby et al. 1971).

The biochemical approach is a different method of studying the possible ways in which a refractory mosquito could prevent parasite development. Haemolymph collected from A. stephensi infected with P. berghei showed net uptake of valine, histidine and particularly methionine, although net loss of arginine compared with haemolymph of non-infected mosquitoes (Mack et al. 1979a). With the same mosquito/parasite system but using extracts of whole mosquitoes, Gad et al (1979b) demonstrated lower concentrations of several other amino acids, namely alanine, asparagine, glycine, and serine, in infected mosquitoes. In addition, the four-fold rise in haemolymph sugar levels observed after a normal bloodmeal was not as great after an infected bloodmeal, although the deficiency was not restricted to any particular sugar of the five identified (Mack et al. 1979b).

The only biochemical study of the differences between refractory and susceptible strains of the same species is that of Frizzi et al (1975) with A. stephensi. After performing assays on a large number of enzymes, they concluded that there was a higher trypsin-like protease activity in midgut homogenates from females refractory to P. gallinaceum. In all the other work mentioned on biochemical requirements of malaria parasites, there are no clear indications of how these requirements are frustrated in refractory mosquitoes.

2.3 POSSIBLE WAYS OF USING REFRACTORINESS

The main motive for the selection of refractory strains of mosquito and the investigation of the inheritance of refractoriness genes is the possibility of their use in the interruption of malaria transmission. Replacement of a population of a pest insect by a type which cannot transmit a disease is preferable to eradication, since it does not leave an empty ecological niche and therefore will not allow susceptible immigrants to immediately re-invade. Other 'desirable' genes, for example insecticide susceptibility or temperature-sensitive mutations, could be introduced into populations by many of the methods discussed below; such replacement is however only the precondition for eradication. Such genes would always be working against a 'fitness gradient' if attempts were being made to replace a type and then eradicate it.

Much of the general theory of genetic control concerns population elimination rather than replacement, and therefore is not relevant in the context of replacement by refractory types. However, several reviews have covered the topic of population replacement as part of general reviews of genetic control methods (Smith & von Borstel, 1972; Davidson, 1974; Pal & LaChance, 1974; Pal & Whitten, 1974; Whitten & Foster, 1975; Fitz-Earle, 1976; Curtis, 1979).

2.3.1 Translocations

The use of translocations in pest control was first considered by Serebrovskii (1940), Curtis (1968a,b) and by Laven (1968). Translocations can potentially be used either for population elimination or replacement, and could be used in either homozygous or heterozygous form. Translocation homozygotes are generally fully fertile, whilst heterozygotes are usually 'semi-sterile', because segregation in the heterozygote gives rise to aneuploid gametes. Therefore the F_1 offspring of matings between a strain homozygous for an autosomal translocation and a wild type are less fertile than the homozygous products of matings within either of the strains. Because of this effect of 'negative heterosis', an unstable equilibrium will occur at relative frequencies of the two types which will depend on their relative viabilities. If the translocation homozygote is fully viable, the equilibrium will occur when each type is in equal numbers,

and any increase in the proportion of one type (e.g. by release of translocation-carrying males) would lead to population replacement by that type. If eradication is being attempted, the proportions of translocated and wild-type insects must be maintained near the equilibrium point, since that is where maximum sterility is obtained (Serebrovskii, 1940; Curtis, 1968b). For replacement, the equilibrium frequency of the translocated type must be exceeded, and the population fertility will return to normal as the replacement proceeds.

Homozygous translocations have been produced in several insects, for example A. culicifacies (Sakai et al. 1979), Ae. aegypti (Lorimer et al, 1972; Rai et al. 1974), C. pipiens (Laven et al. 1971) and others reviewed by Robinson (1976). In most cases, however, few of the homozygotes were viable or had fitness comparable to the wild type, which would be necessary for population replacement to be feasible. The translocated strains must be fairly easy to manipulate in the laboratory, since a refractoriness gene must be closely linked to the translocation breakpoint by position or preferably by an inversion.

In most insects, only autosomal or X-linked translocations can be made homozygous, but in the culicines partially male-linked homozygotes can be made, since sex is determined by a locus on a chromosome pair which carries other genes and which can form crossovers. Such a sex-linked translocation was produced in Ae. aegypti (Lorimer et al. 1972). An attempt was made in the field to replace a wild population with one carrying this translocation (Lorimer et al. 1976). The translocated strain was shown not to have comparable fitness to the wild-type, possibly because it was carrying the recessive sex-linked marker 'red-eye', which was used to follow the progress of the translocation.

If homozygous translocations are not available in pest species, heterozygous translocations may have potential for population control but not for replacement by a refractory type, as where there are no viable homozygotes there is no negative heterosis. Heterozygous translocations have been produced in many species, including A. gambiae s.s. (Krafsur, 1972; Akiyama, 1975); A. stephensi (Aslamkhan & Aaquil, 1970), A. albimanus (Rabbani & Kitzmiller, 1972, 1975) and A. culicifacies. (Baker et al. 1978). Translocations involving the

Y-chromosome are the only usable type, since in a colony started from translocation heterozygote males and normal females, all the males inherit the translocation and it will remain 'pure' in the colony without the necessity of selecting individuals in each generation, and mass rearing is therefore a possibility. Without constant culling, non sex-linked translocations would be selectively eliminated because of the associated semi-sterility.

An attempt was made to eradicate an isolated population of C. pipiens in the field by release of a strain carrying a heterozygous male-linked translocation with 50% fertility (Laven et al. 1972). Although the translocation reached a high frequency immediately after the releases, the semi-sterility declined steadily over the next three years (Cousserans & Guille, 1974). This decline was to be expected given the natural selection operating against the translocation due to its semi-sterility (Curtis, 1975), and illustrates the disadvantages of attempting population eradication rather than replacement.

Y-linked translocations which carry insecticide-resistance genes can be used in conjunction with other genetic control methods aimed at population replacement or eradication, as a means of automatic sex-separation. Such sexing systems have been produced in A. gambiae s.s. (Curtis et al. 1976), A. arabiensis (Curtis, 1978a) and A. albimanus (Seawright et al. 1978). The R70 translocation in A. gambiae s.s. was used to provide males for releases in a cage experiment concerning introduction of genes for insecticide-susceptibility into a population (Prassitisuk, 1979).

Homozygous translocations have good prospects as a system of population replacement, provided that refractoriness gene(s) can be linked to the translocation. Immigration of wild types from outside the replacement area presents problems for the translocation method, as it does for other replacement methods. The effect of immigration might be to stabilise the frequency of a translocated type at a frequency below 100%, preventing complete replacement by a refractory type (von Ende, 1978). The effect that this would have on a control programme would depend on the number of susceptible insects required to maintain transmission.

2.3.2 Compound chromosomes

A strain carrying compound chromosomes, i.e. in which the two homologous arms of each chromosome are attached to the same centromere, is genetically isolated from a chromosomally normal strain, and therefore presents good opportunities for population replacement by negative heterosis (Foster *et al*, 1972). An unstable equilibrium between the compound and normal types will result at a frequency of the compound type determined by the relative fitness of the two types. Since the relative number of viable zygotes for the compound type is 0.25 compared to the wild type, the unstable equilibrium point should occur when the frequency of the compound type is 0.80. Therefore in order for replacement by the compound type to occur, four times the wild population of insects would have to be released. Despite the larger number of released insects theoretically required than for autosomal translocations, compound chromosomes have the advantage that there is no need to worry about linkage of a refractoriness gene to a particular chromosome position, as with the translocation method.

Cage studies with a variety of compound lines in *Drosophila* showed that higher ratios than the theoretical 4:1 (compound:wild-type) were usually required to achieve replacement, although in some cases this was because markers were affecting the fitness of the compound lines (Fitz-Earle, 1975). Compound strains containing recently introduced field material were most successful at replacement in the laboratory and in the field, and were able to transport temperature-sensitive genes into populations (Fitz-Earle *et al*. 1975). A related type of chromosome arrangement found in *D. melanogaster* is that called a compound : free-arm combination (Fitz-Earle & Holm, 1978). Segregation in such strains gives rise to fewer aneuploid gametes than in compound lines. Cage replacement experiments showed that these strains could replace standards at initial ratios as low as 3:1.

It should be remembered that the theoretical frequencies calculated for the unstable equilibrium point are based on a closed population. Field studies show that much higher frequencies than those required in cages are needed when immigration of females is possible (McKenzie,

1976). In population cages, immigration rates of less than 10% virgin migrants did not prevent replacement, but inseminated migrants at even 0.5% did (McKenzie, 1977). The main drawback of the compound chromosome method is that females as well as males must be released to achieve replacement. Possible breakdown of association of the desirable gene with the compound type can also be envisaged, but the effect that this would have once replacement has occurred depends on the relative fitness of the refractory and susceptible types (Foster et al. 1972).

The production of compound chromosomes would not present great difficulty in a species in which a number of whole-arm translocations are available. In pest insects, compounds have so far been synthesised in the sheep blow-fly Lucilia cuprina (Foster et al. 1976) and the onion-fly Hylemya antiqua (van Heemert, 1977).

2.3.3 Cytoplasmic incompatibility

This is the phenomenon of sterility observed in both or only one of the reciprocal crosses between strains or closely-related species of mosquitoes of different geographical origin. It is found in the C. pipiens complex (Laven, 1967a) and the Ae. scutellaris complex (Smith-White & Woodhill, 1954). Cytoplasmic incompatibility is due to failure of gamete fusion after sperm penetration of the egg, and seems to be mediated in the C. pipiens group by the presence or absence of symbiotic rickettsia in the cytoplasm (Yen & Barr, 1973), which are also found in some members of the Ae. scutellaris complex (Beckett et al. 1978).

Cytoplasmic incompatibility has long been recognised as an efficient way of producing sterile males for population eradication. Males of a 'foreign' strain can be used as sterile males against a 'target' strain with which they are incompatible. Since the incompatibility is maternally inherited, backcrosses through the medium of a third strain which is compatible with both 'target' and 'released' strains can produce males with the genome of the wild strain in the background of the foreign cytoplasm. Such males should have no barriers to mating with the wild strain. In an experiment on an isolated population of C. quinquefasciatus in Okpo, Burma, release of such incompatible males reduced the egg hatch in the wild population to zero (Laven, 1967b, 1971).

For population replacement. cytoplasmic incompatibility provides the most extreme case of negative heterosis. Matings between bidirectionally incompatible types are completely sterile at the first generation, unlike homozygous translocated strains, in which the (incomplete) sterility resulting from matings with the wild-type does not take effect until the second generation. On the other hand, matings within each cytoplasmic type are completely fertile, unlike compound chromosome strains, which although completely isolated from wild-type strains, have greatly reduced fertility of within-strain matings. The potential for replacement of a population by an innocuous type using cytoplasmic incompatibility is therefore good. The main drawback, which is also the case for the compound chromosome method, is that females as well as males must be released. Matings between the released insects (which could incorporate the genome of the wild type as before) and the wild insects result in no progeny, and provided the released type is in the majority, replacement by it should occur. The numbers of females released can be small as long as the released males are in the great majority compared to wild-type males (Curtis, 1979). Once replacement has been achieved, the population should be resistant to immigrants, which will make only incompatible matings.

Complete population replacement by cytoplasmic incompatibility depends on full bi-directional incompatibility between the two types, released and wild. Unfortunately, it has been found that incompatibility is partially lost as males of C. quinquefasciatus age (Singh et al. 1976). Polymorphism of cytoplasmic type, which could lead to unidirectional compatibility between a small proportion of the released type with the wild-type, has also been found (Subbarao et al. 1977). Both these phenomena would lead to 'leakage' of genes from one cytoplasm to the other during a replacement process, and might lead to loss of association between a refractoriness gene and a particular cytoplasm.

The principle of transporting a gene, such as one causing refractoriness to filaria, into a population of C. quinquefasciatus by means of cytoplasmic incompatibility has been tested in the laboratory (Curtis & Adak, 1974) and in field cages (Curtis, 1976), using a male-linked translocation as a marker, analogous to a refractoriness gene. The results were encouraging, although the formation of "recombinant"

males possessing the 'foreign' cytoplasm without the translocation was observed. This possible breakdown of association between a 'desirable' gene and its cytoplasmic type due to partial compatibility is a drawback to this method of replacement, although the worst effects of it could be overcome by intermittent releases (Curtis, 1976).

Cytoplasmic incompatibility in the Ae. scutellaris complex is not as clear-cut as in the C. pipiens group, since matings between certain members of the Ae. scutellaris group are unsuccessful for other reasons, such as low insemination rates due to pre-copulatory isolating mechanisms. However, there is evidence of strain differences in crossing-type, as well as species differences (Macdonald, 1976).

One member of the Ae. scutellaris group, Ae. malayensis, is refractory to W. bancrofti (urban periodic form), whilst others (e.g. Ae. polynesiensis, pseudoscutellaris) are susceptible. It is not yet known whether susceptibility in these species operates by a single gene mechanism analagous to the f^m gene of Ae. aegypti. If it does, then it is conceivable that a refractoriness gene from Ae. malayensis could be incorporated into the genome of a susceptible species, which is then backcrossed into a 'foreign' cytoplasm showing bidirectional incompatibility with the susceptible target species (Macdonald, 1976). Such a strain would be ideal for replacement by a refractory type, and the island distribution of members of the Ae. scutellaris complex makes them favourable targets for genetic control projects. However, the barriers of hybrid sterility which operate as well as cytoplasmic incompatibility in this complex may make such a strain difficult to produce in practice.

2.3.4 Hybrid sterility

Crosses between member species of the A. gambiae complex (see Appendix 1A) produce competitive male hybrids which are sterile and fertile female hybrids. Crosses between males of the freshwater species gambiae s.s. or arabiensis and females of the saltwater species melas or merus usually result in F_1 generations almost entirely composed of sterile males. This would seem to provide a very convenient way of introducing sterile males into the field at an early stage of the life cycle (Davidson, 1969a,b).

A field trial was carried out in Pala, Upper Volta to assess the effect of releasing sterile males produced from a cross between A. arabiensis males and A. melas females in eradicating an isolated population of A. gambiae s.s. The sterile males did not mate on any significant scale with the wild females (Davidson et al, 1970). Thus the hopeful results achieved in experimental cage trials must have been due to breakdown of natural mating barriers between the species, which are probably responsible for the very low frequency of hybrids found in the wild (White, 1971). In the Pala experiment, the barriers between species were probably enhanced by the use (for practical reasons) of hybrids between two species against a third species.

The use of sterile hybrids for population eradication in the A. gambiae complex has therefore not fulfilled its promising expectations. Nevertheless, hybrid sterility may yet have applications for the replacement of part of the genome of one population by that of another. This will be considered further below.

2.3.5 Competitive displacement

The process of replacement of one species by another, which frequently occurs naturally, could be used artificially to control disease by replacing a vector species by a non-vector. If two species can cross-mate, but produce sterile hybrids, then competitive displacement would operate by negative heterosis. If they do not cross-mate, then the replacement process will only work if the released species is superior in direct ecological competition with the wild species.

When an attempt was made to replace Ae. polynesiensis, the principal vector of W. bancrofti, by Ae. albopictus, a closely related non-vector, on a Pacific atoll, none of the three strains of Ae. albopictus released survived for more than four years, and it was never found in large numbers relative to Ae. polynesiensis (Rosen et al, 1976). The reason for the failure of replacement was probably that the strains were ill-adapted to field conditions, but also possibly due to the relatively low numbers of Ae. albopictus released. Although Ae. polynesiensis females will mate with Ae. albopictus males in small cages, under large cage conditions Ae.

polynesiensis females are more receptive to males of their own species (Ali & Rozeboom, 1973). So few albopictus were released in the experiment of Rosen et al (1976) that they would have rarely encountered another albopictus with which to mate.

2.3.6 Meiotic drive

Meiotic drive is any change in the mechanics of meiosis which causes a heterozygote to fail to produce two types of gametes in the expected 1:1 ratio (Sandler and Novitski, 1957). It has been extensively studied in Drosophila, in which there are both sex-ratio distorting and autosomal meiotic drive factors. In mosquitoes, sex-ratio meiotic drive systems have been discovered in Ae. aegypti, in which the drive factor is termed M^D (Hickey & Craig, 1966), and in C. pipiens (Sweeny & Barr, 1978). In Ae. aegypti, the effect of M^D requires a sensitive m- allele in females, and it causes the sensitive m- chromosome to fragment at meiosis (Newton et al, 1976). Most populations of Ae. aegypti contain at least some m alleles resistant to M^D (Wood & Newton, 1977; Suguna et al, 1977).

Population replacement presents problems for a sex-linked meiotic drive system, especially for diseases in which the female only is the vector, such as malaria or filariasis. If a refractoriness factor were tightly associated with the distorter locus (e.g. with M^D), then it would never get into the females. If it were not closely linked, then crossing-over would ensure that it would be inherited by females, but it would not go to fixation. This was illustrated by an experimental attempt to replace a wild-type cage population of Ae. aegypti with one homozygous for red-eye (re), analagous to a desirable gene, by releasing males of a strain carrying re linked to the M^D locus. The frequency of re in both sexes was increased but crossing-over prevented it going to fixation (Wood et al, 1977).

Problems encountered with sex-linked distorter factors might be overcome by releasing the refractoriness gene linked to the resistant m in females together with M^D. In the presence of M^D, resistant m genes are favoured. Provided that the released m is more resistant than any in the target wild population, there would be efficient replacement of the wild m chromosomes by the released ones. A non-

sex-linked distorter factor would however be preferable for transporting refractoriness genes into vector females.

2.3.7 Dilution

In anopheline vectors of malaria, neither cytoplasmic incompatibility nor meiotic drive is available as a transporting system, so we are left with the possibilities of translocations or hybrid sterility. So far, homozygous autosomal translocations have not been produced in A. gambiae s.l., and hybrid sterility would only be useful if there is a factor controlling refractoriness on the X-chromosome. The other alternative is 'dilution', i.e. replacement of the wild type by sheer weight of numbers. 'Dilution' is the method employed in the present work, being the only possibility available for A. gambiae s.s.

The 'dilution' method would work equally well for desirable characters which are controlled by polygenes as for those which are controlled by single genes. Release of both males and females would ensure more rapid rise in frequency of the desirable character, but, for malaria vectors, release of males only would still lead eventually to replacement without raising the proportion of harmful females during the releases. Once replacement has been achieved, it is likely to be 'resistant' to immigration unless the refractoriness gene or genes confer reduced fitness.

At present the only example of replacement of a pest insect by a non-pathogenic type by dilution is in an agricultural pest of wheat, the Hessian fly (Mayetiola destructor). Virulence in this pest is genetically determined at several loci and each pair of alleles for virulence can overcome a specific pair of alleles for resistance in wheat. Avirulence at a locus is dominant to its allele for virulence (Gallun, 1977). In greenhouse tests and in field cages, the virulent Race B was eradicated by releases of both sexes of the Great Plains race, which is unable to stunt resistant soft wheat and cannot survive on it (Foster and Gallun, 1972, 1973). Eradication of wild population by releases of the Great Plains race onto screened-off sections of a field showed that it had good mating competitiveness (Foster, 1977). Since the Great Plains race cannot survive on the soft type of wheat, it left an empty niche which could then be re-invaded. In the case of mosquitoes refractory to malaria, the intention is that the refractory type

would be able to survive and fill the niche.

The use of 'dilution' as a means of introducing 'desirable' genes for insecticide susceptibility into a resistant population was tested by Prasittisuk (1979). He found that releases of DDT susceptible males into a resistant population were able to reduce the level of resistance in the population and keep it low, even when selection for DDT resistance was applied to the population.

3. MATERIALS AND METHODS

3.1 MOSQUITOES

3.1.1 Strains of A. gambiae s.s.

LD5: The white-eyed, P.berghei-refractory strain selected by Al-Mashhadani (1976). The forerunner of this strain (LSW) was derived from a population from Sokoto, Nigeria in 1951 and selected by Mason (1967) for the recessive sex-linked gene white-eye (w) controlling pigment in the larva, pupa and adult.

16cSS: A strain collected in Lagos, Nigeria in 1950 which is homozygous for the recessive gene for dieldrin-susceptibility.

4Y: A strain obtained by crossing LD5 males to 16cSS females, backcrossing F_1 females to LD5, and then reselecting for normal eyes, dieldrin-susceptibility, and refractoriness to P. berghei by rearing single families, as described in the Results section.

156L: A strain derived in the same series of crosses as 4Y. It is therefore also homozygous for dieldrin-susceptibility and normal eyes.

Both 4Y and 156L underwent further selection for refractoriness to P.v. nigeriensis during the work, leading to the strains 4Y1, 156L1, 4Y5.4, 156L4.2 etc. as explained in the Results section.

R70: This strain carries the R70 translocation between a part of the chromosome carrying the semi-dominant gene for dieldrin-resistance and the Y-chromosome (Curtis et al, 1976). When males of this strain are mated to homozygous dieldrin-susceptible females, treatment of the offspring with 0.01 ppm dieldrin as 1st instar larvae or 0.4% dieldrin as adults kills all the females whilst the males survive. Because of the translocation, the R70 strain is semi-sterile. There is

about 0.25% crossing-over between the dieldrin-resistance gene and the translocation breakpoint, leading to loss of linkage between the dieldrin-resistance gene and the Y-chromosome.

R704Y: A strain derived by outcrossing males of the R70 strain to 4Y or 156L females, or females of strains derived from them, for 30 generations. After outcrossing, first instar larvae were treated with 0.01 ppm dieldrin to provide males for the next generation.

PB: The normal-eyed, P. berghei susceptible strain which was selected by Al-Mashhadani (1976). The forerunner of this strain, PALA, was collected at Pala, Upper Volta in 1963, and is homozygous for dieldrin-resistance.

PYN: A strain derived from PB by selecting for increased susceptibility to P.y. nigeriensis. Subsequent generations of selection for susceptibility led to the strains designated PYN 1,2,3 etc., the number indicating the number of generations of mass selection.

FAJARA: This strain is kept at the MRC Laboratories, Fajara, The Gambia. It was originally isolated from Kerbasenti and Bansang and has been colonized for at least three years.

SOK W: A strain derived from the population from Sokoto, Nigeria, which also gave rise to LD5. It is homozygous for w.

A: A w-eyed strain derived from SOK W by selecting for dieldrin-resistance.

3.1.2 Strain of A. stephensi

BEECH: This strain was originally derived from Delhi, India, in about 1947. It is 100% susceptible to P. berghei and P.y. nigeriensis. and was used as a control in the rodent malaria experiments to check the infectivity of the parasites. It was also used for routine cyclical transmission of the parasite before preservation in liquid nitrogen.

3.2 MALARIA PARASITES

P. yoelii nigeriensis N67

This strain was first isolated in 1967 in Western Nigeria from *Thamnomys rutilans* (Killick-Kendrick *et al.* 1968) (see Appendix 1C). Two mice infected with *P. y. nigeriensis* N67 were received from Dr. D. Walliker, Protozoan Genetics Unit, University of Edinburgh, on 23 November 1976. The history of the strain from 1976 is shown in Fig. 1.

P. falciparum

Blood containing *P. falciparum* parasites was obtained from patients with naturally acquired infections who attended the Out Patients Department of the MRC Laboratories, The Gambia.

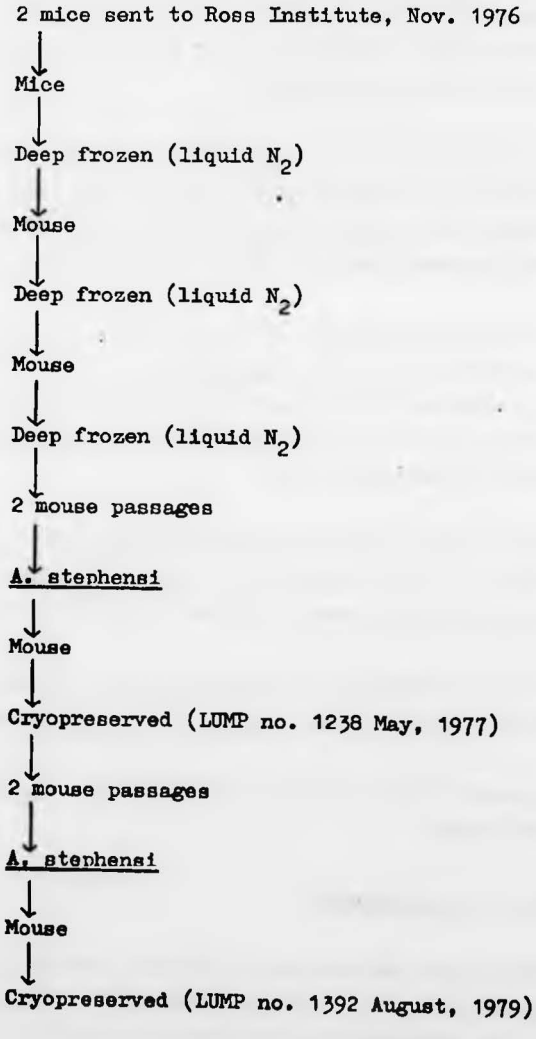
3.3 REARING OF MOSQUITOES

Eggs were left in the egg-bowls to hatch for two days. The first instar larvae were distributed into 30 cm diameter white plastic bowls together with a piece of grass and soil to encourage growth of micro-organisms. The density per bowl was about 300 to 400 larvae per 2 l of tap water. The larvae took approximately ten days to develop at 26°C. Finely ground Farex was given once a day to the young larvae and twice a day to older larvae. Pupae were either picked individually from the bowls, or the whole bowl was strained and the pupae and larvae placed in a cage to complete development. Sometimes adults were allowed to emerge under nets covering the bowls and transferred to cages by mouth aspirator.

When virgin adults were required, males and females were separated on the first day of emergence before mating could occur. When single families were required, gravid females were isolated two days after blood-feeding in 8 cm by 3 cm glass vials, the lower $\frac{1}{3}$ of which were lined with filter paper, containing about one cm^3 of tap water. Egg-batches from single females were allowed to hatch in the tubes and then transferred to enamel bowls 10 cm in diameter for rearing.

The stock colonies of adult mosquitoes were kept in 30 cm cube cages in an insectary at 25 to 28°C and a relative humidity of about 80%.

Fig. 1. History of Plasmodium yoelii nigeriensis (N67) in the Ross Institute



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The stock colonies of adult mosquitoes were kept in 30 cm cube cages in an insectary at 25 to 28°C and a relative humidity of about 80%.

Guinea pigs for blood feeding and egg bowls were provided twice a week. 20% glucose solution was provided at other times in feeders with a lint wick which were changed weekly. The lighting routine was 12 hours light and 12 hours dark.

3.4 MAINTENANCE OF MALARIA PARASITES

After the P. y. nigeriensis strain was received, it underwent several blood passages and also periods of preservation in liquid nitrogen, mixed with heparinised glycerol in saline, as described by Al-Mashhadani (1976). These stored samples were used in early experiments. To avoid the necessity for continuous cyclical transmission and to minimise variation between experiments, stabilates were made by the method of Lumsden et al. (1973) from mice with sporozoite-induced infections. Blood from a mouse on day 7 of the sporozoite-induced infection was added to 0.1 ml heparin solution on ice. Glycerol was added to give a final concentration of 7.5% and mixed. The blood mixture was drawn into a syringe from which capillaries were filled. They were heat-sealed and then slow-cooled to -70°C overnight, before being placed in the liquid nitrogen. 66 capillaries were made from a mouse with 25% parasitaemia (LUMP number 1238). 25 capillaries were made from a mouse with 20% parasitaemia (LUMP number 1392).

3.5 ROUTINE INFECTION PROCEDURES

3.5.1 P. y. nigeriensis

These techniques are based on those of Shute & Maryon (1966), Wéry (1968), Killick-Kendrick (1971) and Al-Mashhadani (1976). Mice of the second blood passage were routinely used for mosquito infection. Occasionally it was necessary to use the third passage.

Infected blood stored in capillaries was recovered by transferring the capillary directly from the liquid N_2 to water at 37°C. After a few minutes the contents of the capillary were put into 1 ml sterile 0.85% saline. 0.2 to 0.4 ml was then injected into each of two mice. T.O. (Theiler's original) mice were usually used although Balb c were used for some of the work. No differences were noted between the behaviour of the parasite in these two strains.

Blood films were taken from the tail of the mouse on day 4 or 5 after

infection, fixed with methanol and stained for 20-30 minutes in 10% Giemsa (Hopkin & Williams) in buffered distilled water at pH 7.2. Blood from the tail of a mouse with about 5 to 10% parasitaemia was mixed with heparinised normal saline, usually in a 1 in 10 dilution, and amounts of diluted blood were innoculated intra-peritoneally into two or three mice such that each received approximately 10^6 parasitized red blood cells.

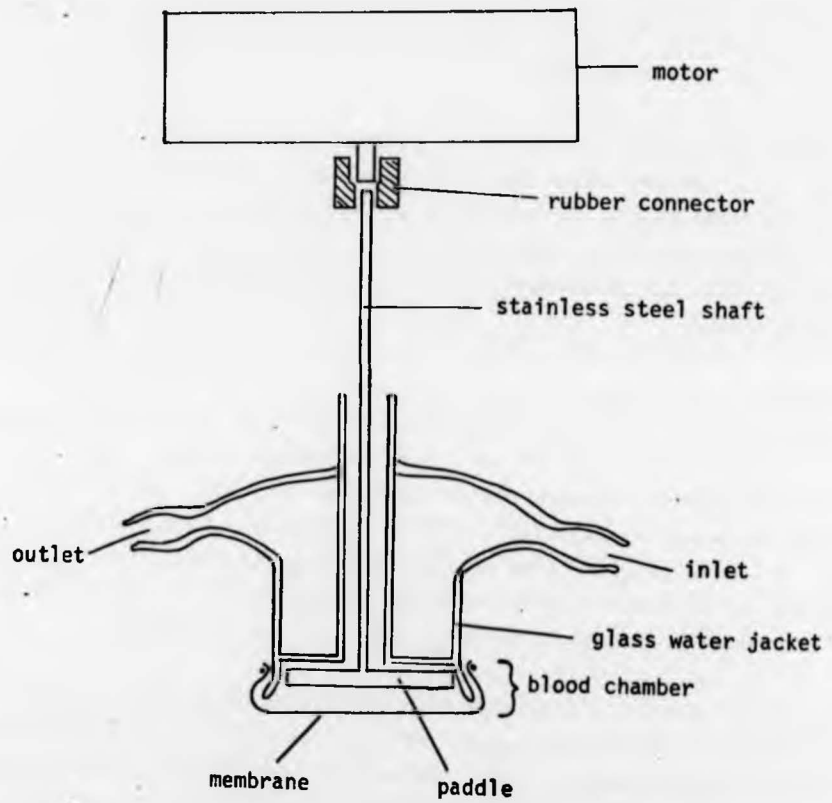
Mosquitoes were fed on day 3 after passage (day of passage = day 0) except on a few occasions when they were fed on day 2 or 4. On day 3, a mouse was chosen for infection which had a parasitaemia of 5-10% and 1-20 gametocytes of each sex in 50 fields (1 field = 750 red blood cells). At this stage of the infection the parasitaemia is still rising.

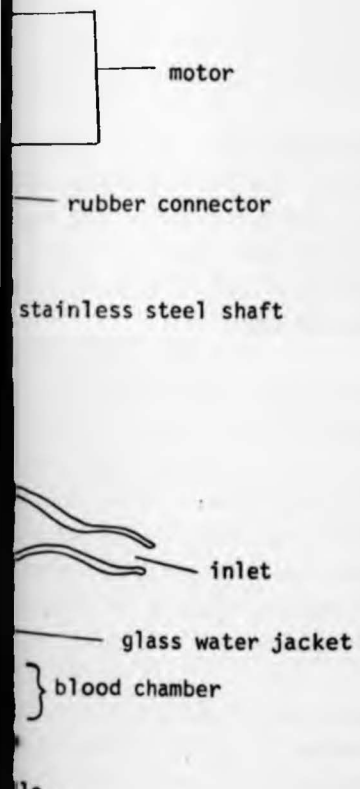
Originally, mice chosen for infection were immobilised by attaching adhesive tape to each leg and pinning it to a cork board with the animal on its back. This seems to be a cruel procedure, however, and in later experiments the anaesthetic 'Hypnorm' (Janssen) was used which causes the mouse to lie still without constraint. Anaesthesia of about one hour resulted from an injection i.p. of 0.2-0.3 ml of a 10% solution of Hypnorm in normal saline. Mosquitoes fed readily within 5-10 minutes on anaesthetised mice placed on top of a cage.

Mosquitoes reared for infection were allowed to emerge into recently bleached 20 or 25 cm cube cages. Movement of adults by aspirator was avoided to avoid damaging them. Mosquitoes were usually infected when 4 to 6 days old, although difficulties of synchronising two or more strains meant that occasionally adults as young as two days or as old as ten days were used. Removal of the glucose feeder a few hours before the feed made the mosquitoes hungrier and also ensured that the abdomen was clear of glucose allowing complete engorgement to take place.

After the bloodmeal, unfed and partially fed females were removed, and usually the males, except when inseminated fed mosquitoes were required. The mosquitoes were then maintained at 24 $\pm$ 1°C and at greater than 90% R.H. by covering the cages with damp lint and plastic bags. If eggs were required, a second bloodmeal was given, and the gravid females isolated for egg-laying.

Fig.2. The membrane feeding apparatus.





Dissections were usually carried out on day 7 (occasionally 6 or 8) after the infective feed, which was on day 0. At 24°C oocyst development is not complete until day 8 and oocyst counts made before that date will therefore be accurate. Stomachs were dissected out into normal saline and examined at low power, under which oocysts on both sides of the stomach could be seen by focussing up and down on the flattened stomach. In some of the earlier experiments batches of mosquitoes were left until the salivary glands were infected (days 9 to 11 and later) and the glands were then also dissected.

3.5.2 P. falciparum

Infections with human malaria were done solely by membrane feeding. The apparatus was modified after Wade (1976) and is shown diagrammatically in Fig. 2. The glass feeders were of two types: 'large'; 2.5 cm in diameter, and 'small'; 1.5 cm in diameter, taking 1.5-2.0 cm^3 and 0.5-0.8 cm^3 of blood respectively. Each feeder was equipped with a four-bladed (large feeders) or two-bladed (small feeders) paddle of stainless steel which stirred the blood at 30 r.p.m. to prevent settling of the red cells. Baudruche membrane was moistened with saline and stretched over the feeders. Water from a water-bath at 40°C circulated through the feeders to warm the blood.

P. falciparum gametocyte carriers were identified by thick blood films stained with Field's stain. Gametocytes were counted per 1000 wbc and a white cell count done. Blood samples of 0.5 to 8 cm^3 were obtained in the late morning by venipuncture and placed immediately into heparinized Universal tubes or 'Vacutainers' (approximately 10 units heparin per ml). The blood was placed in the feeders within five munutes of bleeding and presented to A. gambiae s.s. which had been starved overnight and which were allowed to feed for 10 to 40 minutes. Mosquitoes fed well if they were at a density of about 300 per 20 cm cube cage in a darkened room. Extreme care was taken to remove all unfed or partially fed mosquitoes.

Otherwise, mosquito maintenance before and after the infective feed was as described for P. y. nigeriensis, except for a few points. The mosquito larvae were reared at the ambient temperature (about 22-32°C). The stock and infected adults were kept in the same insectary at

27 ± 4°C and 80-90% R.H. At this temperature the oocysts of P. falciparum do not mature before day 9. Dissections were usually done on day 7 or 8. On a few occasions mosquitoes were kept beyond day 9, and the glands were then also dissected.

On two occasions, exflagellation of P. falciparum gametocytes was delayed by immediately diluting the blood in 50 vols. of S.A. (suspended animation) medium (modified from Carter and Nijhout, 1977). S.A. medium consisted of 10 mM tris, 145 mM NaCl and 10 mM glucose at pH 7.4. To recover the gametocytes, the red cells were washed in fresh S.A. medium and resuspended to approximately 40% haematocrit in inactivated FCS or bicarbonate-free serum (Nijhout & Carter, 1978).

3.6 MATING COMPETITION TESTS

Mating competitiveness of strains was assessed by using radiation sterilization as a convenient marker to identify which type of male had mated with a particular female. In each test, one- or two-day old males of one strain were irradiated with 9 krad of γ-rays from a Co^{60} source, and then placed in a 45 cm cube cage with an equal number of the same age males of the other strain and an equal number of virgin females from one of the strains. After 8 days for mating, during which time two bloodmeals were given, females were isolated for egg-laying. Sterile egg batches (5 or fewer hatched larvae) were the result of matings to the irradiated strain.

The mating efficiency of males with females from the same strain (for instance, at different cage densities) was assessed by examining the spermathecae of females caged with the males for various lengths of time. If present, sperm are easily seen inside the spermatheca, or beside the ruptured spermatheca, under phase contrast.

3.7 CAGE REPLACEMENT EXPERIMENT

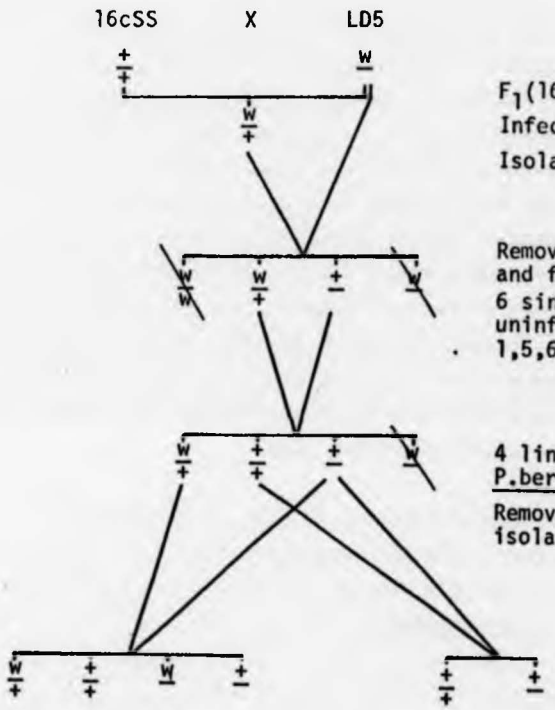
A cycling cage population of A. gambiae strain PYN1 was established in a 1 m cube cage at 24-28°C and about 75% R.H. in an insectary provided with a 12 hours light/12 hours dark light regime with simulated 'dawn and dusk' lighting. 175 males and 175 females were put into the cage each week, either as pupae (in which case account was taken of failed emergents) or usually as virgin adults. Guinea pigs for blood-

feeding and egg-bowls for oviposition were provided twice a week. The eggs were removed from the cage for rearing.

The cage population was the target for the release of males of a Plasmodium-refractory strain in an attempt to 'dilute out' the Plasmodium-susceptibility, which was 100% in the female population at the start. It was originally intended to release males of the translocation-carrying strain R704Y, to test the effects of its semi-sterility on the population replacement and also to reduce the workload by automatic sexing; however the semi-sterility, together with general lack of fitness meant that extreme difficulty was experienced in rearing large numbers of R704Y. The refractory strains 4Y1 and 156L4.2 were used instead, although neither was completely refractory. Adults and pupae were sexed by hand for three separate periods of releases of males over two years in numbers up to three times the normal number of males returned to the cage.

The susceptibility of the cage population to P. y. nigeriensis was monitored once every two weeks (except during one period of seven months) by rearing some of the eggs to provide adults for infection, together with the control A. stephensi.

Fig.3. Production of strains 4Y and 156L from LD5 and 16cSS.



F_1(16cSS X LD5)
Infected with P.berghei.
Isolated single females.

Removed white males and females.
6 single families from uninfected mothers 1,5,6,4,2,7.

4 lines infected with P.berghei: (1,5,6),4,2,7.
Removed white males, isolated single females.

Families producing some white males discarded.

Families from uninfected mothers producing all + offspring kept.
Lines 156L and 4Y.

56L from LD5 and 16cSS.

F_1(16cSS X LD5)
Infected with P.berghei.
Isolated single females.

Removed white males
and females.
6 single families from
uninfected mothers
1,5,6,4,2,7.

4 lines infected with
P.berghei: (1,5,6),4,2,7.
Removed white males,
isolated single females.

+

lies from uninfected
ers producing all +
pring kept.
s 156L and 4Y.

4. RESULTS

4.1 EXPERIMENTS WITH RODENT MALARIA

4.1.1 Susceptibility of strains of A. gambiae s.s.

The refractory strain available at the start of the work was LD5, the white-eyed, dieldrin-susceptible strain which was selected by Al-Mashhadani (1976) for refractoriness to P. berghei. The first task was to eliminate the white-eye gene (w) from this strain, since this mutant seemed likely to have serious effects on mating competitive ability in the proposed population replacement experiment, especially as white-eyed mosquitoes can be seen to be less active than normal.

The crossing scheme by which the strains 4Y and 156L were produced is shown in Fig. 3. The crossing scheme was designed to produce normal-eyed strains which had on average 75% of the autosomal genes of the refractory strain LD5. During the crossing scheme selection for refractoriness was attempted. LD5 males were crossed to females of the dieldrin-susceptible strain 16cSS. The F_1 females, which were all heterozygous for white-eye, were backcrossed to LD5 males, and were also infected with P. berghei. The females were isolated for egg-laying. Six out of the eight females dissected were found to be uninfected, and separate lines set up from them. An attempt to infect the backcross progeny failed, so, after elimination of white-eyed males and females, the sibs were allowed to mate and the lines were infected with P. berghei at the next generation. At this point three of the lines were pooled to form one. After infection, single females of the four remaining lines were isolated for egg-laying, and the eggs of those females which were uninfected when dissected, and which produced all normal-eyed offspring when mated to normal-eyed males, were kept. Two of such families were 156L and 4Y.

Difficulty was encountered in infecting even A. stephensi control mosquitoes with P. berghei, and in maintaining the insectary temperature as low as the required 21°C. For this reason P. y. nigeriensis was used instead. 100% infections were soon established in the A. stephensi controls once the correct infection conditions had been established for P. v. nigeriensis, and the number of oocysts per mosquito was usually more than 200.

Table 1. Susceptibility to P. y. nigeriensis of strain 4Y and its derivative 4Y1

Strain	Date	N*	% infected	G.M.**	G.M.(pos)***	
4Y	7.6.77	8	62.5	4.9	23.8	a
4Y1	28.11.77	13	38.5	1.6	3.6	
"	17.2.78	6	33.3	2.4	14.3	
"	4.5.78	46	26.2	2.0	12.9	
"	7.7.78	12	25.0	2.4	34.4	b
"	21.7.78	25	20.0	1.4	5.4	c
Average 4Y1		102	26.5			

* N = number of mosquitoes dissected
** G.M. = geometric mean of oocyst number
*** G.M.(pos) = geometric mean of oocyst number amongst positive mosquitoes.

a : A. stephensi BEECH controls were 92.0% infected
b : Control mosquitoes all died before dissection
c : Controls were 95.0% infected

In all other experiments, controls were 100% infected.

Table 2. Susceptibility of lines derived by single family selection from 4Y1

Strain	No. of exp'ts	Date	N*	% infected
4Y1	1	11/77	13	38.5
4Y1.2	2[a]	1/78	18	27.8
4Y1.4	2[a]	1/78	23	8.7
4Y1.8	1[a]	1/78	4	25.0
4Y1.9	3[a]	1/78-4/78	11	9.1
4Y1.10	2[a]	1/78	34	38.2

*N = Number of mosquitoes dissected
a : In one experiment, A. stephensi BEECH controls were 90.0% infected
In all other experiments controls were 100% infected.

eriensis of strain 4Y and its

% infected	G.M.**	G.M.(pos)***	
62.5	4.9	23.8	a
38.5	1.6	3.6	
33.3	2.4	14.3	
26.2	2.0	12.9	
25.0	2.4	34.4	b
20.0	1.4	5.4	c
26.5			

ed
number
cyst number amongst positive

92.0% infected
e dissection

re 100% infected.

ived by single family selection

te	N*	% infected
/77	13	38.5
/78	18	27.8
/78	23	8.7
/78	4	25.0
/78-4/78	11	9.1
/78	34	38.2

ECH controls were 90.0% infected
100% infected.

The susceptibility of strains 4Y and 156L to P. y. nigeriensis could then be assessed. Susceptibility was measured in two ways:

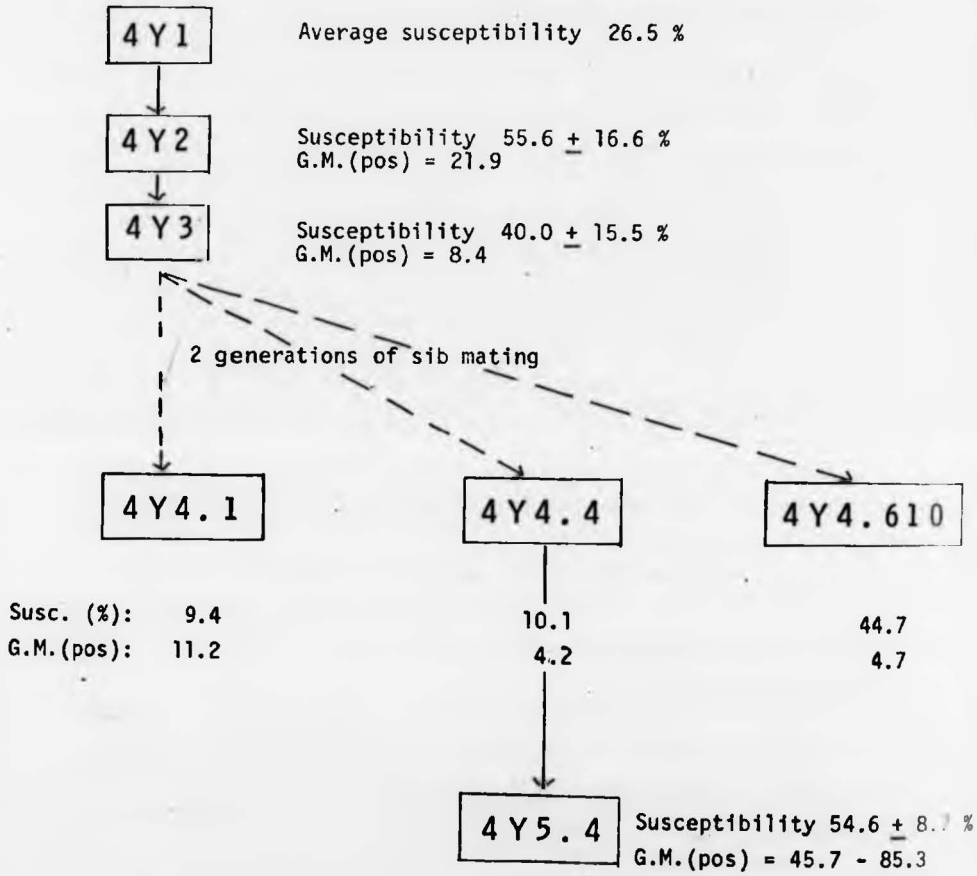
i) the percentage of a batch of mosquitoes infected: the criterion of "infected" was either no oocysts on the gut if dissections were made before day 8, or no oocysts or sporozoites if preparations of both guts and glands were made after day 8.

ii) the intensity of infection: this is expressed as the geometric mean of oocyst number, based on the total sample of mosquitoes (G.M.), and as the geometric mean of oocyst number amongst mosquitoes positive for infection (G.M. (pos)). Oocyst numbers of more than 200 were difficult to count accurately and were scored as 250 for the purposes of calculating the geometric mean.

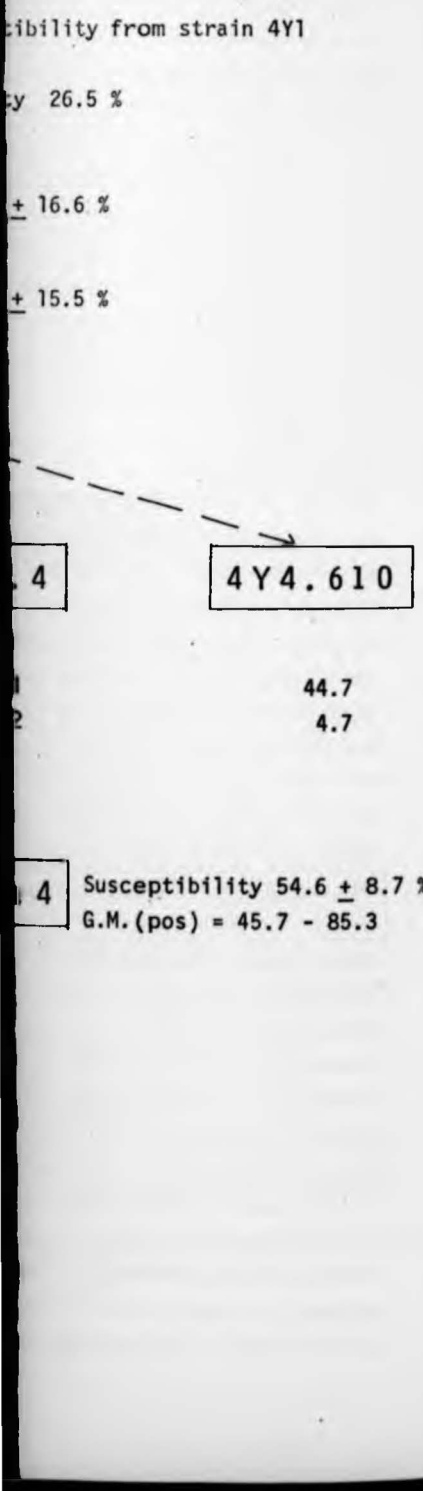
Neither 4Y nor 156L was found to be refractory. 4Y had a susceptibility of over 60%, although the number of oocysts was always far less than in the controls. Two tests of the susceptibility of 156L gave conflicting values of 57% and 17%. One generation of mass selection for refractoriness was immediately carried out on each strain, by isolating females for egg-laying before dissection, and rearing eggs only from refractory mothers. This gave rise to strains termed 4Y1 and 156L1 respectively.

4Y1 was used and tested at intervals from November 1977 to August 1978, and had a fairly stable susceptibility of about 27%. (Table 1). The strain was hampered by poor fitness which made it difficult to rear. Two unsuccessful attempts to purify the refractoriness in 4Y1 were made by rearing inbred lines from single refractory females. In the first attempt, in which the offspring of the selected females were infected with P. y. nigeriensis, the lines died out, probably because of the excessive stress caused by inbreeding and malaria infection. However, the dissection results did indicate between-family variation in susceptibility to P. y. nigeriensis (Table 2) before the families died out. Disregarding family 4Y1.8 because of the small number dissected, the difference in proportion infected between the other families was found to border on statistical significance (Heterogeneity $\chi^2_5 = 7.42$, $0.1 > P > 0.05$). In the second attempt to rear inbred lines, the families died out after a few generations of inbreeding, before they could be tested for susceptibility.

Fig. 4. Selection for reduced susceptibility from strain 4Y1



All controls 100% infected



There was an interval of seven months, from August 1978 to March 1979, during which the susceptibility of 4Y1 was not tested. After this period, there was a large increase in the susceptibility of 4Y1 to about 54%. Whether the change was due to contamination or to selection back towards susceptibility because of low fitness associated with refractoriness, is not known. Whatever the reason, the fluctuating susceptibility of 4Y1 was a major problem in the work, since it was the strain selected for the cage replacement experiment described below.

After March 1979, a further programme of selection for refractoriness was carried out. Firstly two generations of mass selection were done, with infection of females with P. y. nigeriensis at each generation and pooling of offspring from those females which were uninfected; the susceptibilities at each generation are shown in Fig. 4, where the number following '4Y' indicates the number of generations of selection which have been carried out (4Y2, 4Y3 etc.). Then single family selection was carried out, by isolating single females at generation 4Y3, when the susceptibility was 40% (Fig. 4). The lines set up from uninfected females of 4Y3 were not infected until after two generations of inbreeding, to allow numbers to build up. The results of the infections of each line of the resulting 4Y4 strain are shown in Fig. 4. As this Figure shows, the single family selection appeared to give very encouraging results, and there was a significant difference between the susceptibility of the different 4Y4 lines (Heterogeneity $\chi^2_2 = 20.75$, $P < 0.0001$). Two of them (4Y4.1 and 4Y4.4) appeared to have lower susceptibility than had previously been obtained in the 4Y strain. One of the lines, 4Y4.4 with a susceptibility of 10.1 ± 3.6%, was selected to continue the strain. It underwent a further generation of mass selection with P.y. nigeriensis and was then termed 4Y5.4. Three separate infections of 4Y5.4 unfortunately revealed that the susceptibility was still as high as that of 4Y1 (Fig. 4). The reason for the low levels of infection obtained at the previous generation (the 4Y4 single families shown in Fig. 4) must have been that the conditions for infection were not right in this experiment. This is shown by the fact although all the control BEECH mosquitoes were infected, they had a very low intensity of infection (G.M.(pos) = 8.4).

Strain 156L1, derived from 156L, was not tested as often as 4Y1, but it had an average susceptibility of 28% over the period February 1978

Table 3. Susceptibility to *P. y. nigeriensis* of strain 156L and its derivative 156L1

Strain	Date	N*	% infected	G.M.**	G.M.(pos)***	
156L	13.6.77	7	57.1			
156L	26.7.77	6	16.7			
156L1	28.2.78	10	0.0	-	-	
"	4.5.78	9	33.3	4.1	70.4	
"	7.7.78	15	46.7	1.8	3.6	a
"	24.5.79	16	25.0	1.6	5.7	
"	20.8.79	78	28.2	2.2	16.6	
Average 156L1		128	28.1			

* N = Number of mosquitoes dissected
** G.M. = geometric mean of oocyst number
*** G.M.(pos) = geometric mean of oocyst number amongst positive mosquitoes

a : *A.stephensi* BEECH control: all died before dissection
In all other experiments, controls were 100% infected.

Fig. 5. Selection for reduced susceptibility from strain 156L1.

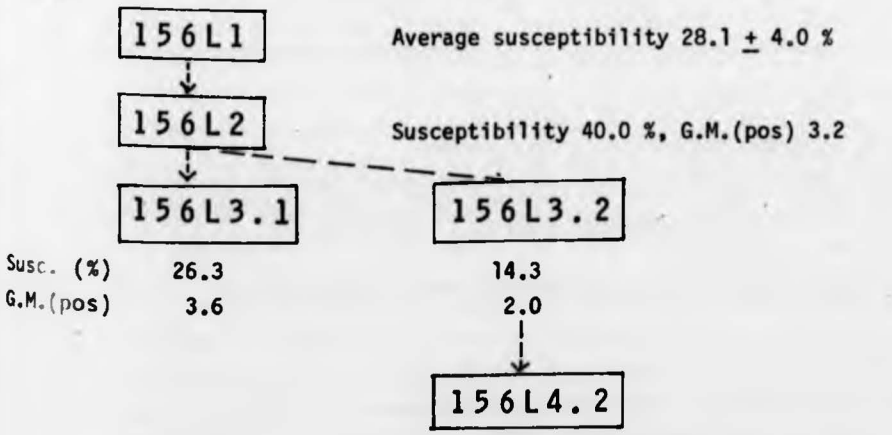


Table 4. Susceptibility to *P. y. nigeriensis* of strain PB and strains derived from it

Strain	No. of expts	Date	N*	% infected	G.M.**	G.M.(pos)***
PB	3[a]	6/77	29	93.0		
PYN1	1	8/77	12	83.3	15.5	26.8
"	1[b]	11/77	5	100.0	14.7	14.7
"	1	12/77	8	100.0	109.5	109.5
"	1	2/78	10	100.0	58.2	58.2
PYN1	1	3/79	17	70.6	16.2	51.7
PYN2	1	4/79	9	55.6	15.2	134.3
PYN3	1	5/79	11	54.6	5.0	19.3
PYN4	1	7/79	21	100.0	55.1	55.1
PYN5	4	8/79-10/79	41	78.1		

* N = Number of mosquitoes dissected

** G.M. = geometric mean of oocyst number

*** G.M.(pos) = geometric mean of oocyst number amongst positive mosquitoes

a : In one experiment, *A. stephensi* BEECH controls were 92.0% infected

b : In this experiment, " " " " 93.8% "

In all other experiments, the controls were 100% infected.

igeriensis of strain PB and strains

N*	% infected	G.M.**	G.M.(pos)***
29	93.0		
12	83.3	15.5	26.8
5	100.0	14.7	14.7
8	100.0	109.5	109.5
10	100.0	58.2	58.2
17	70.6	16.2	51.7
9	55.6	15.2	134.3
11	54.6	5.0	19.3
21	100.0	55.1	55.1
41	78.1		

cted
t number
pocyst number amongst positive mosquitoes
1 BEECH controls were 92.0% infected
" " " 93.8% "
rols were 100% infected.

to August 1978 (Table 3). 156L1 remained unchanged until March 1979, unlike 4Y1 which showed a marked increase in susceptibility over this period, and this fact suggests that refractoriness in 156L1 is not associated with low fitness.

After March 1979, a further programme of selection was carried out on 156L1. This was to provide a 'reserve' refractory strain in case selection to reduce the susceptibility of 4Y1 was unsuccessful. It was thought preferable to continue experiments with 4Y1 if at all possible; however problems encountered with lack of refractoriness in this strain as described above suggested that another strain might be required. After one generation of mass selection leading to 156L2, single females were isolated and two lines set up from uninfected mothers (156L3.1 and 156L3.2). The susceptibility of each line is shown in Fig. 5. One of the lines, 156L3.2 with a susceptibility of 14.3 ± 9.4%, was chosen to continue the strain. It underwent one further generation of mass selection resulting in strain 156L4.2, with an average susceptibility in two separate experiments of 15.2 ± 6.6%.

Strain PB was the P. berghei - susceptible strain of Al-Mashhadani (1976), and when tested with P. y. nigeriensis was found to be highly, although not completely, susceptible to this parasite (Table 4). One generation of mass selection to give the strain PYN1 was enough to raise the susceptibility to 100%, where it remained from August 1977 until February 1978. PYN1 was used to set up the 'target' population in the cage replacement experiment described below.

When tested in March 1979 after an interval of seven months without testing or selection, the susceptibility of PYN1 was found to have decreased to 70.6 ± 11.1% (Table 4). Since a fully susceptible strain was required for crosses between refractory and susceptible strains, selection was re-started on PYN1. Four generations of mass selection, leading to the strain designated PYN5, had little effect on the susceptibility, although in the PYN4 generation the sample tested had 100% susceptibility (Table 4). Four separate infections of PYN5 showed that its average susceptibility was 78.1 ± 6.5%, i.e. little better than that of PYN1 in March 1979.

Fig. 6. Percentage susceptibility to P.y.nigeriensis of A.gambiae s.s. strains 4Y,156L and PYN and their derivatives.

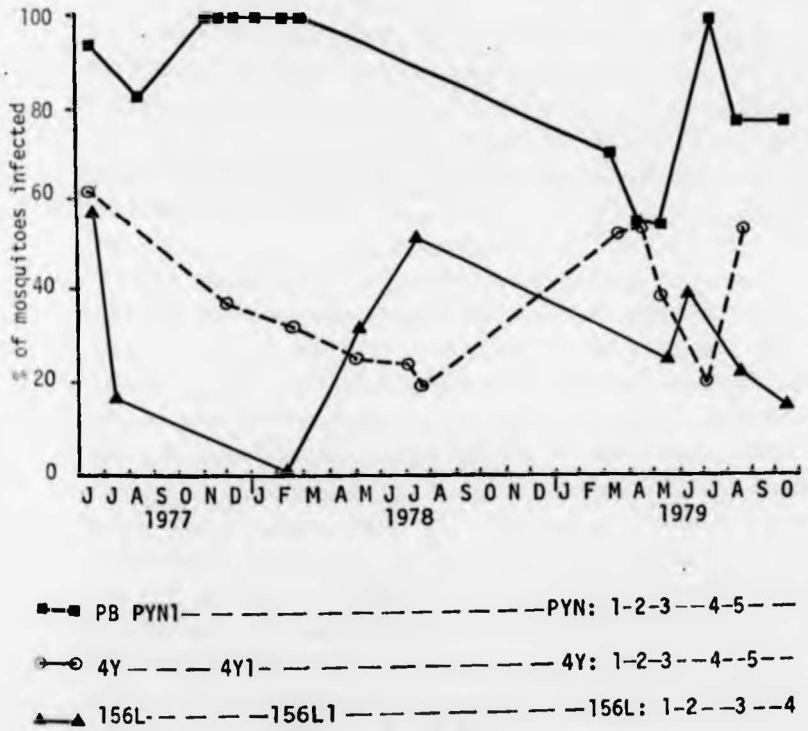
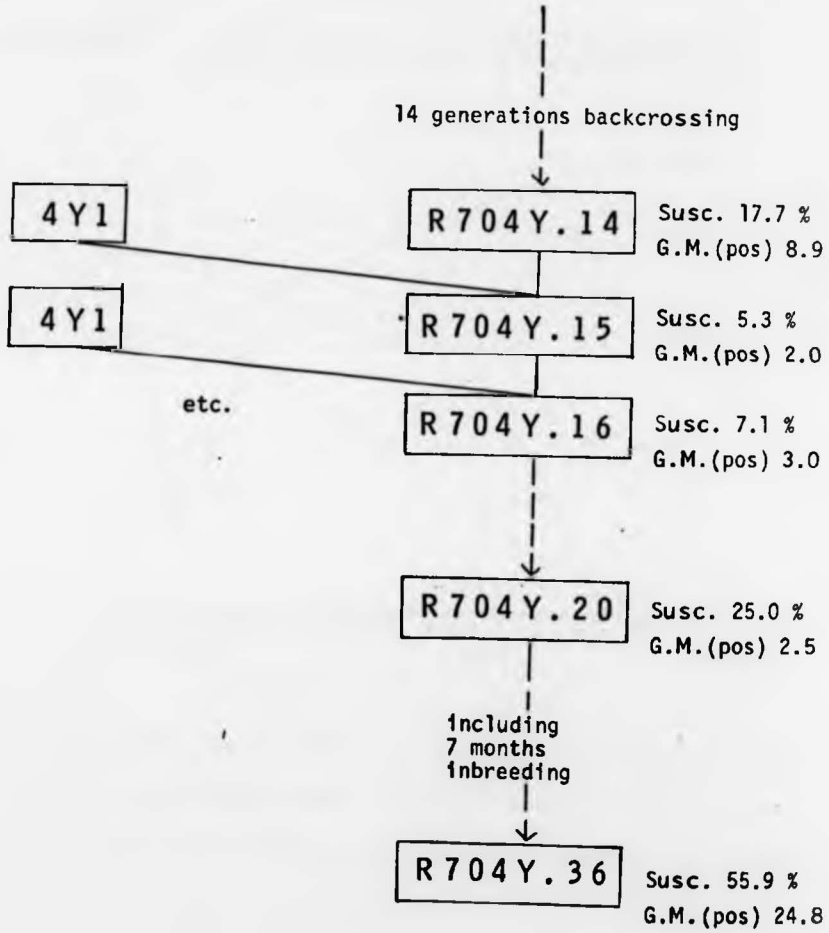


Fig. 7. Introduction of the R70 sexing system into the refractory line, and susceptibility of the R704Y strain.



em into the refractory line,
ain.

erations backcrossing

04Y.14 Susc. 17.7 %
G.M.(pos) 8.9

04Y.15 Susc. 5.3 %
G.M.(pos) 2.0

04Y.16 Susc. 7.1 %
G.M.(pos) 3.0

04Y.20 Susc. 25.0 %
G.M.(pos) 2.5

ncluding
months
nbreeding

704Y.36 Susc. 55.9 %
G.M.(pos) 24.8

The levels of susceptibility of strains 4Y, 156L, PB and their derivatives to P. y. nigeriensis over a 28 month period are shown in Fig. 6. Although the susceptibility fluctuated in the three strains, the 4Y and 156L lines always had a lower percentage susceptibility than PYN. During the early part of the work (i.e. from October 1977 to July 1978) there was a marked difference in the percentage susceptibility: PYN1 had an average susceptibility of 100% whilst that of 4Y1 was 26.5% (S.N.D. = 6.50, $P < 0.0001$), and 156L1 also had a significantly lower susceptibility of 29.4% (S.N.D. = 5.30, $P < 0.0001$). There was also a difference between the strains with regard to the oocyst numbers. Over the same period the G.M.(pos) for PYN1 was 14.7-109.5, whilst for 4Y1 it was 3.6-14.3. The range in 156L1 was larger (0-70.4), and overlapped with PYN1.

The R70 strain produced by Curtis et al (1976) enables batches of males to be produced easily in large numbers, because it carries a gene for dieldrin-resistance translocated onto the Y-chromosome. Treatment of larvae with 0.01 ppm dieldrin kills all the females, whilst rearing of larvae without treatment allows both sexes to survive and the females can then be tested for susceptibility to malaria. In order to maintain the sexing ability of the strain, males must be crossed at each generation to homozygous susceptible females (i.e. their sibs or other females). The reason for the inclusion of dieldrin-susceptibility in the refractory strains 4Y and 156L (Fig. 3) was so that females could be crossed to R70 males. Backcrossing for several generations should combine most of the genome of the refractory strain with the translocated Y-chromosome. The intention was to use this strain to provide refractory males for releases in the proposed cage replacement experiment.

Backcrossing of R70 strain males to females of 4Y (occasionally 156L) or their derivatives 4Y1 and 156L1 continued for 36 generations, the number of backcrosses being indicated in the name of the strain, e.g. R704Y.15, R704Y.20 etc. (Fig. 7). At some generations, R704Y females were reared and infected with P. y. nigeriensis; results obtained in these infections are shown in Fig. 7, which illustrates that by generation 14 of backcrossing a strain as refractory as 4Y1 had been produced. On one occasion, the R704Y strain at generation 15 was crossed to susceptible PYN1 females to investigate the inheritance of refractoriness, the results of which are discussed below.

Table 5. Susceptibility to P. y. nigeriensis of strains R704Y and PYN1 and the F_1 and F_2 from a cross between them

Strain	No. of exp'ts	Date	N	% infected	G.M.(pos)	Oocyst distribution 0	0-10	11-50	51-200	>200
R704Y (gens 15-20)	4	7/77-12/77	58	12.1	4.3	51	6	1	0	0
PYN1	4[a]	8/77-12/77	100	98.0	76.4	3	11	20	29	37
F_1 (PYN1 x R704Y.15)	1	8/77	13	30.8	5.5	9	4	0	0	0
F_2 (PYN1 x R704Y.15)	1	9/77	33	81.8	7.8	6	14	11	1	1

a : In one experiment, A. stephensi BEECH controls were 93.8% infected. In all other experiments, the controls were 100% infected.

Table 6. Susceptibility to P. y. nigeriensis of progeny of backcrosses set up from F_1 (PYN1 x R704Y)

Cross type	Female parent	Male parent	No. of exp'ts	Date	N	% infected	G.M.(pos)	Oocyst distribution 0	0-10	11-50	51-200	>200
1	(PYN1 x R704Y)	PYN1	2[a]	9/77	18	77.8	22.3	4	5	4	5	0
6	R704Y	(PYN1 x R704Y)	2[a]	9/77	10	10.0	2.0	9	1	0	0	0
7	(PYN1 x R704Y)	R704Y	2[a]	9/77	17	52.9	21.6	9	2	3	3	0

a : In one experiment, A. stephensi BEECH controls were 71.4% infected. In all other experiments, the controls were 100% infected.

Table 6. Susceptibility to *P. y. nigeriensis* of progeny of backcrosses set up from F_1 (PYN1 x R704Y)

Cross type	Female parent	Male parent	No. of exp'ts	Date	N	% infected	G.M.(pos)	Oocyst distribution 0	0-10	11-50	51-200	>200
1	(PYN1 x R704Y)	PYN1	2[a]	9/77	18	77.8	22.3	4	5	4	5	0
6	R704Y	(PYN1 x R704Y)	2[a]	9/77	10	10.0	2.0	9	1	0	0	0
7	(PYN1 x R704Y)	R704Y	2[a]	9/77	17	52.9	21.6	9	2	3	3	0

a : In one experiment, *A. stephensi* BEECH controls were 71.4% infected. In all other experiments, the controls were 100% infected.

The R704Y strain was hampered by the translocation, which approximately halves the egg hatch (Curtis *et al*, 1976), and it seemed also, not surprisingly, to suffer from the low fitness observed in 4Y1. The egg hatch of R704Y was 21.1% (sample of 2266 eggs) and it proved very difficult to rear large numbers of males.

After generation 30 of backcrossing, the R704Y strain was allowed to inbreed. If the dieldrin-resistance gene were fully linked to the Y-chromosome by the translocation, the strain should retain its sexing ability indefinitely. However, there is a known frequency of crossing-over between the dieldrin-resistance locus and the translocation breakpoint, such that about 0.25% of females are recombinant heterozygotes carrying a dieldrin-resistance gene (Curtis *et al*, 1976).

After seven months of inbreeding the sexing ability of the strain had been lost. Although this was easily rectified by rearing of single families and selecting only those in which all males survived and all females died after dieldrin treatment, the susceptibility level to *P. y. nigeriensis* was also found to have increased to 56% (see Fig. 7). Whether this was due to contamination or to selection against refractoriness is not clear, but in any case the strain was discarded.

4.1.2 Inheritance of refractoriness

Two attempts were made to study the inheritance of refractoriness. In neither case was it possible to make crosses between completely refractory and susceptible lines.

Crosses and backcrosses were made between the strains R704Y.15 (partially refractory) and PYN1 (susceptible) in August and September 1977. The results of these crosses are shown in Table 5, together with data on the average susceptibilities of the parental strains from several experiments done during July-December 1977. The results indicate that the F_1 is intermediate in susceptibility, as measured by percentage of guts and glands infected, although it resembles more closely the refractory parent. The oocyst numbers in dissected mosquitoes are not distributed normally, and the results for intensity of infection are therefore expressed as frequencies in particular classes, and the geometric mean of oocyst number is also quoted. With regard to the intensity of infection, the F_1 can be

Table 7. Susceptibility to P. y. nigeriensis of strains 4Y1 and PYN1, and F_1s from the reciprocal crosses between them

Strain	No. of exp'ts	Date	N	% infected	G.M.(pos)	Oocyst distribution 0	0-10	11-50	51-200	>200
4Y1	4[a,b]	2/78-7/78	85	24.7	12.2	64	9	9	3	0
PYN1	6	12/77-2/78	149	100.0	113.6	2	9	21	35	82
F_1(PYN1 x 4Y1)	3[b]	4/78-7/78	55	60.0	16.8	25	15	6	6	3
F_1(4Y1 x PYN1)	2[b]	4/78-7/78	54	77.8	44.8	12	8	11	12	11

a : In one experiment, all A. stephensi BEECH controls died before dissection

b : In one experiment, controls were 95.0% infected.

In all other experiments, the controls were 100% infected.

Table 8. Susceptibility to P. y. nigeriensis of progeny of backcrosses set up from reciprocal F_1s of (4Y1 x PYN1)

Cross type	Female parent	Male parent	No. of exp'ts	Date	N	% infected	G.M. (pos)	Oocyst distribution 0	0-10	11-50	51-200	>200
1	(PYN1 x 4Y1)	PYN1	1	4/78	10	90.0	94.9	1	2	0	2	5
2	(4Y1 x PYN1)	PYN1	1	4/78	13	84.6	174.5	2	0	0	3	8
3	PYN1	(PYN1 x 4Y1)	1	4/78	27	100.0	164.1	0	0	3	5	19
4	PYN1	(4Y1 x PYN1)	1	4/78	26	88.5	126.3	3	0	4	5	14
5	4Y1	(4Y1 x PYN1)	1	4/78	3	66.7	16.5	1	0	2	0	0

Cross type	Female parent	Male parent	No. of expts	Date	N	% infected	G.M. (pos)	Oocyst distribution 0	0-10	11-50	51-200	>200
1	(PYN1 x 4Y1)	PYN1	1	4/78	10	90.0	94.9	1	2	0	2	5
2	(4Y1 x PYN1)	PYN1	1	4/78	13	84.6	174.5	2	0	0	3	8
3	PYN1	(PYN1 x 4Y1)	1	4/78	27	100.0	164.1	0	0	3	5	19
4	PYN1	(4Y1 x PYN1)	1	4/78	26	88.5	126.3	3	0	4	5	14
5	4Y1	(4Y1 x PYN1)	1	4/78	3	66.7	16.5	1	0	2	0	0

seen to resemble much more closely the refractory parent. The F_2 generation has a high susceptibility compared to the F_1, although the intensity of infection remains low whilst showing the greater spread of oocyst number which is expected on almost any model involving segregating Mendelian genes.

In only three of the four backcrosses possible with the F_1 (PYN1 X R704Y) did any mosquitoes survive for dissection, and even in those cases the numbers were small. Nevertheless, as can be seen in Table 6, the backcrosses to the susceptible male parent (cross type 1) gave higher percentage susceptibility than the F_1, whilst backcrosses to the female refractory type (cross type 6) gave progeny with lower percentage susceptibility than the F_1. Backcross type 7 to the male refractory parent gave the anomalous result that the progeny had higher susceptibility than the F_1.

If refractoriness were controlled by a single gene which is dominant, then we would expect to find a bi-modal distribution of oocyst number in the offspring of backcrosses to the susceptible parent. From cross 1 in Table 6 there is certainly a more even spread of oocyst number in the progeny than is the case for the other two crosses, but the distribution is not bimodal. It should be noted, though, that there is wide spread of oocyst number in the parent susceptible strain (Table 5), so that the bimodality of the oocyst distribution in the backcross progeny would not be expected to be clear-cut.

In the second series of crosses, reciprocal crosses between strains 4Y1 and PYN1 were carried out between April and July 1978. The results are given in Table 7, which also gives average susceptibilities of the parent strains from experiments performed between December 1977 and July 1978. The F_1 results for the percentage infected are intermediate between the two parental susceptibilities, although susceptibility of the F_1 from the cross 4Y1 female X PYN1 male is higher than from the reciprocal cross, and this difference is statistically significant (S.N.D. = 2.01, $P < 0.05$). The geometric mean of oocyst number is also higher in the first type of cross, although in both cases it is closer to that obtained for the refractory parent.

Backcrosses were set up with both F_1s, but there were few survivors

Table 9. Observed percentage susceptibilities of F_1, backcrosses and F_2, and expectations if refractoriness is dependent on a gene or genes showing exactly intermediate dominance.

1. Crossing PYN1 (98% susceptible) with R704Y (12% susceptible)

Cross type	Female parent	Male parent	Observed % susceptible	Expected % susceptible	
				If sex-linked	If autosomal
	F_1 generation				
	PYN1	R704Y	30.8	55.0	55.0
	Backcrosses				
1	(PYN1 x R704Y)	PYN1	77.8	76.5	76.5
6	R704Y	(PYN1 x R704Y)	10.0	55.0	33.5
7	(PYN1 x R704Y)	R704Y	52.9	33.5	33.5
	F_2 generation				
	Intercrossing (PYN1 x R704Y)		81.8	76.5	55.0

2. Crossing PYN1 (100% susceptible with 4Y1 (25% susceptible)

Cross type	Female parent	Male parent	Observed % susceptible	Expected % susceptible	
				If sex-linked	If autosomal
	F_1 generation				
	PYN1	4Y1	60.0	62.5	62.5
	4Y1	PYN1	77.8	62.5	62.5
	Backcrosses				
1	(PYN1 x 4Y1)	PYN1	90.0	81.25	81.25
2	(4Y1 x PYN1)	PYN1	84.6	81.25	81.25
3	PYN1	(PYN1 x 4Y1)	100.0	100.0	81.25
4	PYN1	(4Y1 x PYN1)	88.5	62.5	81.25

ilities of F_1, backcrosses and F_2,
iness is dependent on a gene or
diate dominance.

h R704Y (12% susceptible)

served % eptible	Expected % susceptible If sex-linked	If autosomal
30.8	55.0	55.0
77.8	76.5	76.5
10.0	55.0	33.5
52.9	33.5	33.5
81.8	76.5	55.0

h 4Y1 (25% susceptible)

served % ceptible	Expected % susceptible If sex-linked	If autosomal
0.0	62.5	62.5
7.8	62.5	62.5
0.0	81.25	81.25
4.6	81.25	81.25
0.0	100.0	81.25
3.5	62.5	81.25

after infection of the progeny (Table 8). Results were only obtained for one backcross to the refractory parent (cross type 5), but with a sample of only 3 mosquitoes. All of the four backcrosses to the susceptible parent gave progeny with higher susceptibility than the F_1, as expected, and there are indications of bimodality in the oocyst distributions in all of them except cross type 3.

Table 9 shows the observed susceptibilities in the two sets of crosses, and the results expected given the hypothesis of exactly intermediate dominance of refractoriness, assuming either sex-linkage or autosomal control by a single gene or additive polygenes. Comparing the observed results with those expected, it can be seen that there are some results which do not fit either hypothesis (namely the results of backcross types 6 and 7, and the different results for the reciprocal F_1s from the cross of 4Y1 X PYN1 mentioned earlier). The result for the F_2 (PYN1 X R704Y) suggests sex-linkage, as does the result for backcross type 3, whilst the result of backcross type 4 is more indicative of autosomal control of refractoriness.

4.1.3 Effect of infection with *P. y. nigeriensis* on mosquito survival

During infection experiments it was noticed that there was a large and unpredictable level of mortality in mosquitoes after the infective feed. If the level of mortality is different in the refractory and susceptible strains, the differential death rates between the infective feed and the day of dissection could influence the results in experiments involving crosses between refractory and susceptible types, or in the cage replacement experiment. Whilst there is no doubt that humidity and cage density influence the mortality, three experiments were carried out to test whether the infection, or the presence of infected blood in the mosquito's stomach, was correlated with higher mortality after a blood feed. Each experiment involved comparing mortality in samples of mosquitoes fed on infected and uninfected mice. The same two mice were used for all mosquito strains in a particular experiment.

In the first experiment, mosquitoes of the F_2 generation from the cross (PYN1 X R704Y) were used, together with *A. stephensi* BEECH controls. Mortality was checked at intervals until day 12. The results (Table 10) showed that in the case of *A. gambiae s.s.*, a significantly greater mortality was observed after the infected blood-meal, and was apparent by day 2 as well as on day 12. In the case of

Table 10. Mortality of *A. gambiae s.s.* and *A. stephensi* after infected or uninfected bloodmeal (*P. y. nigeriensis*)

Strain	Mouse	No. fed	Proportion dead 2 days after feed	S.N.D.	Proportion dead 12 days after feed	S.N.D.
F_2 (PYN1 x R704Y) (*A.gambiae*)	Infected	85	0.29	5.10***	0.59	3.70***
	Uninfected	77	0.00		0.30	
BEECH (*A.stephensi*)	Infected	99	0.12	1.72 N.S.	0.86	5.78***
	Uninfected	95	0.05		0.47	

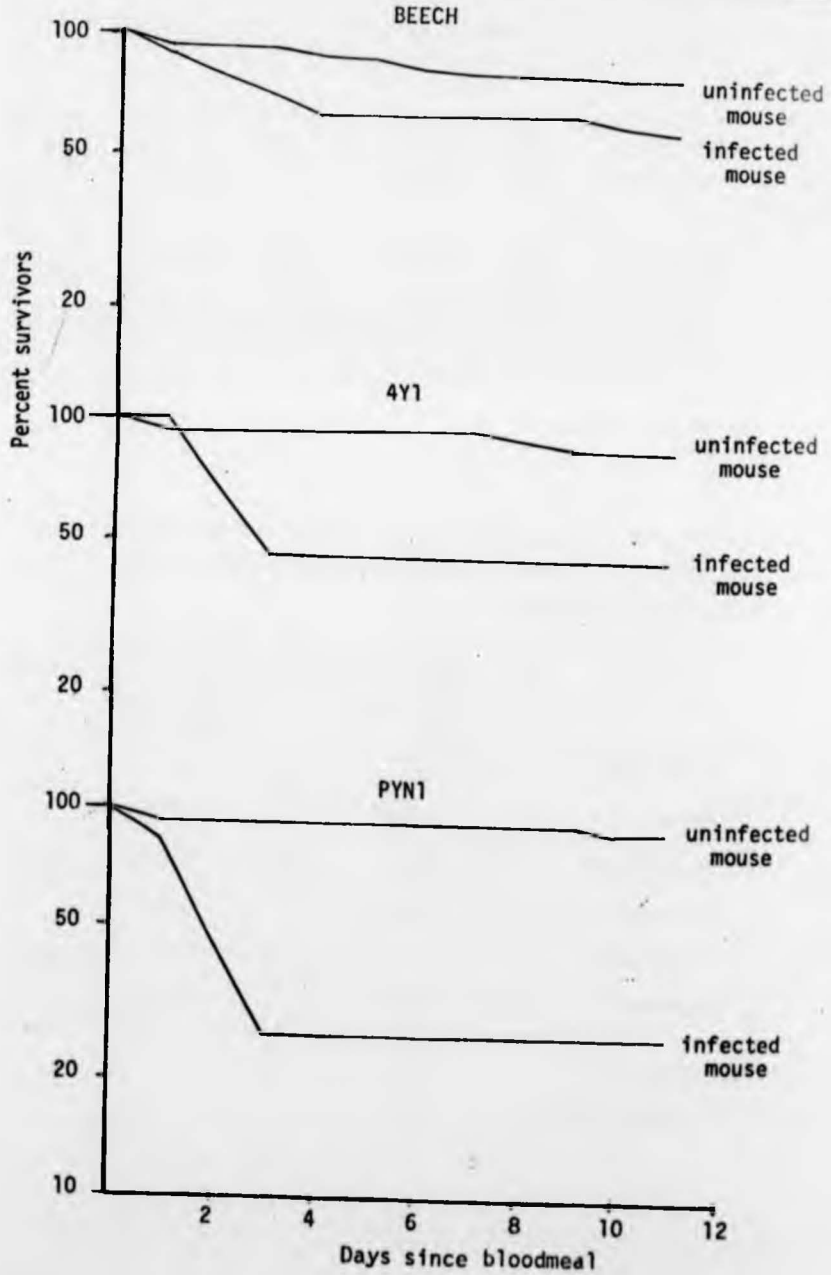
*** P < 0.001
N.S. Not significant

Table 11. Mortality of *A. gambiae s.s.* strains R704Y and PYN1 and *A. stephensi* after infected or uninfected bloodmeal (*P. v. nigeriensis*)

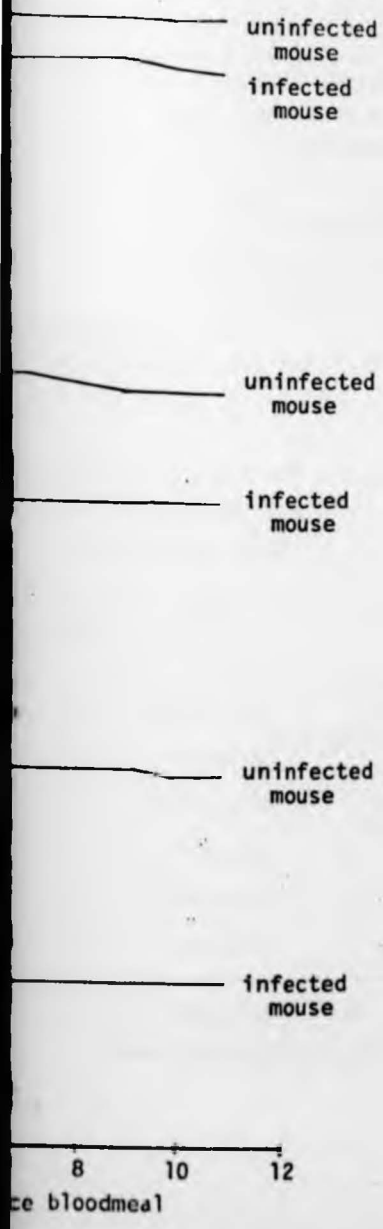
Strain	Mouse	No. fed	Proportion dead 2 days after feed	S.N.D.	Proportion dead 12 days after feed	S.N.D.
BEECH (*A.stephensi*)	Uninfected	65	0.077	4.29***	0.554	1.45 N.S.
	Infected	51	0.412		0.686	
R704Y gen.20	Uninfected	16	0.063	1.54 N.S.	0.313	0.88 N.S.
	Infected	15	0.267		0.467	
PYN1	Uninfected	48	0	5.69***	0.104	6.81***
	Infected	43	0.512		0.814	

*** P < 0.001
N.S. Not significant

Fig. 8. Survival of A.gambiae s.s. strains 4Y1 and PYN1 and A.stephensi BEECH after infected or uninfected bloodmeal (P.y.nigeriensis).



ns 4Y1 and PYN1 and A.stephensi
ed bloodmeal (P.y.nigeriensis).



A. stephensi, mortality by day 12 after an infected bloodmeal was also greater but there was no difference on day 2.

The first experiment therefore indicated that in general there is higher mortality after an infected bloodmeal. The second and third experiments tested specifically whether refractory and susceptible types had different mortality rates after infected bloodmeals, as might be expected given that the refractory strain becomes infected to a much lesser extent than the susceptible one. The effect of differential mortality rates would not have been apparent in experiment 1 since the samples fed on both the infected and uninfected mouse were a mixture of genotypes.

In the second experiment, strains R704Y.20 (susceptibility 25.0%), PYN1 (100%) and BEECH (A. stephensi, 100%) were used. Mortality was noted at intervals until dissection, and the results are shown in Table 11. At day 2 after a bloodmeal, BEECH and PYN1 strains showed significantly higher mortality in those batches fed on the infected mouse. By day 12 the infected PYN1 mosquitoes still showed a significantly higher mortality but this was not the case for BEECH. The R704Y strain showed a trend similar to BEECH, but the numbers were small and the difference was not statistically significant. There was found to be no difference between the proportions dead by day 2 in the refractory and susceptible strains (S.N.D. = 1.64, N.S.), although there was a difference by day 12 (S.N.D. = 2.59, $P < 0.01$). Day 12 is however later than the time at which batches of mosquitoes are usually dissected.

In the third experiment, strains 4Y1 (susceptibility at this time 26.5%), PYN (100%) and BEECH (100%) were compared. The pattern of mortality after an infected or uninfected bloodmeal can be seen in Fig. 8. This shows that mortality was again greater after an infected bloodmeal, and also that most of the deaths occurred between days 1 and 3 after the bloodmeal. The greater mortality after an infected bloodmeal was found to be statistically significant for all strains on days 3 and 11 (Table 12). Although Fig. 8 suggests that mortality might have been greater in the susceptible strain PYN1, in fact there was no significant difference between the proportions of 4Y1 and PYN1 which died (S.N.D. = 1.54, N.S.).

Table 12. Mortality of *A. gambiae s.s.* strains 4Y1 and PYN1, and *A. stephensi*, after infected or uninfected bloodmeal (*P. y. nigeriensis*)

Strain	Mouse	No. fed	Proportion dead 3 days after feed	S.N.D.	Proportion dead 11 days after feed	S.N.D.
BEECH (*A.stephensi*)	Uninfected	51	0.078	3.14**	0.235	2.29*
	Infected	66	0.318		0.439	
4Y1	Uninfected	21	0.048	3.54**	0.143	2.75**
	Infected	20	0.550		0.550	
PYN1	Uninfected	30	0.067	5.71***	0.133	5.14***
	Infected	43	0.744		0.744	

* P <0.05
** P <0.01
*** P <0.001

Fig. 9. Survival of A.gambiae s.s. after infected bloodmeal.

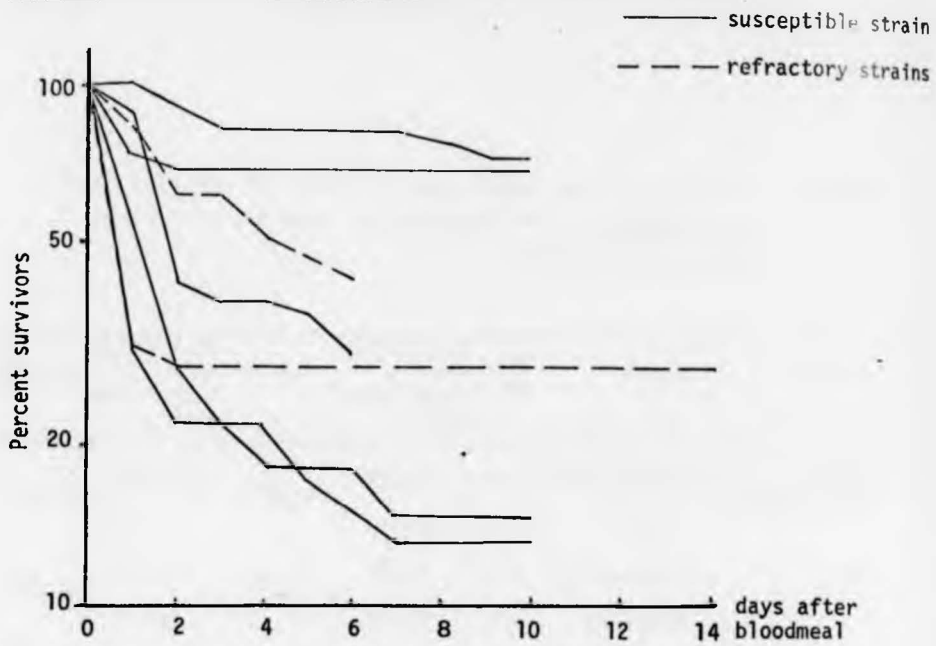
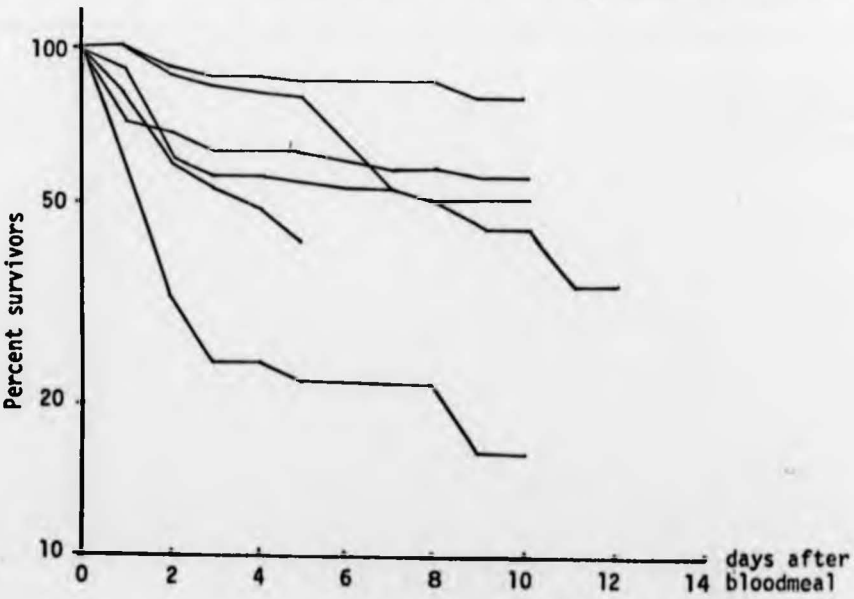
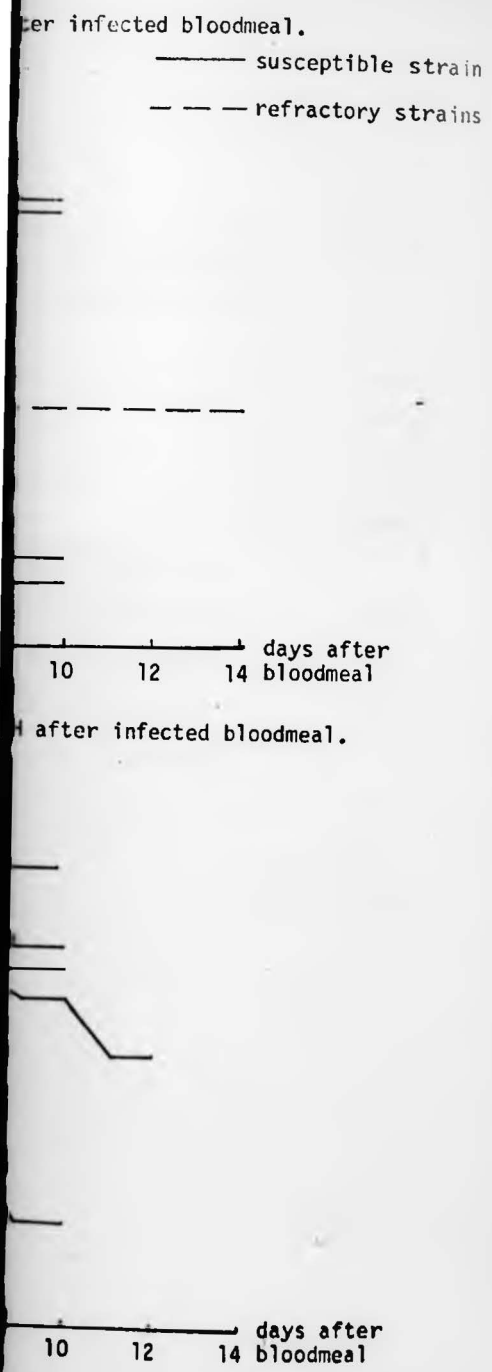


Fig. 10. Survival of A.stephensi BEECH after infected bloodmeal.





The second and third experiments demonstrate that both the refractory (R704Y.20 or 4Y1) and susceptible (PYN1) strains suffer from increased mortality to approximately the same extent after an infected bloodmeal.

When mortality was studied by counting deaths in batches of A. gambiae s.s. and A. stephensi infected for other experiments, it was again found that there was usually a large mortality in the first two or three days following the bloodmeal, although the extent of the mortality varied widely between experiments. The mortality rate then usually levelled off until the day of dissection. Data obtained from typical experiments are shown in Figs. 9 and 10, expressed as percentage survival after a bloodmeal. The effect of early mortality followed by a gradually increasing survival rate is more marked for A. gambiae s.s. (Fig. 9) than for A. stephensi (Fig. 10).

The fact that mortality after the infective feed is not confined only to susceptible strains, and must therefore not be due to oocyst formation but to the presence of infected blood or ookinetes in the stomach, is reassuring since it allows confidence to be placed in the results of infections of populations containing a mixture of genotypes e.g. the experimental cage population. It seems unlikely that susceptible types are dying at a greater rate than refractory types before the day of dissection.

4.2 REPLACEMENT BY REFRACTORY TYPES

4.2.1 Cage replacement experiment

In this experiment, a cycling population of the P. y. nigeriensis susceptible strain PYN1 was set up with overlapping generations in a 1 m cube cage, as a model of an isolated wild population. Releases of males of the refractory strains 4Y1 or 156L4.2 were made into the cage population to introduce refractoriness genes. The progress of the replacement was followed by testing the susceptibility of samples of mosquitoes reared from eggs laid by the cage population.

4.2.1.1 Preliminary data

Investigations were made of the survival and egg production of the strains PYN1 (the 'target' strain) and 4Y1 (the strain intended for

Fig. 11. Survival of males and females of A.gambiae s.s. strain PYN1.

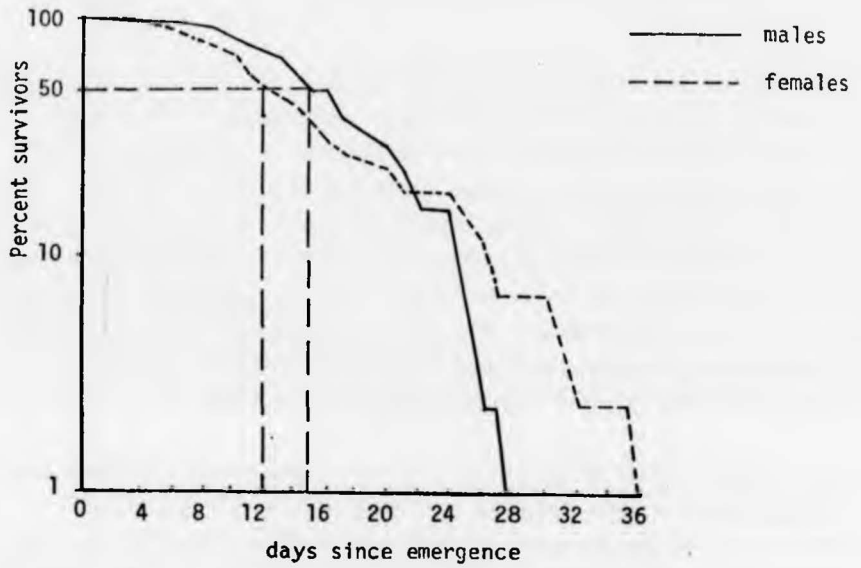
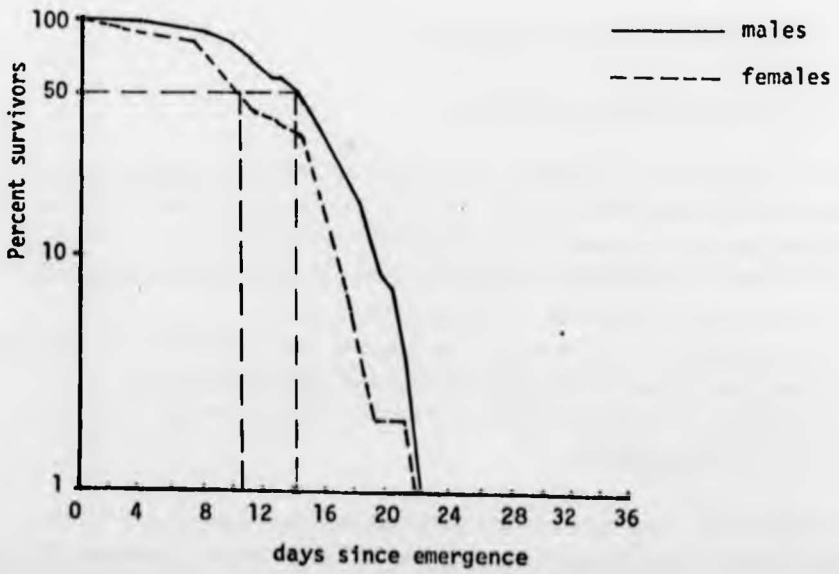


Fig. 12. Survival of males and females of A.gambiae s.s. strain 4Y1.



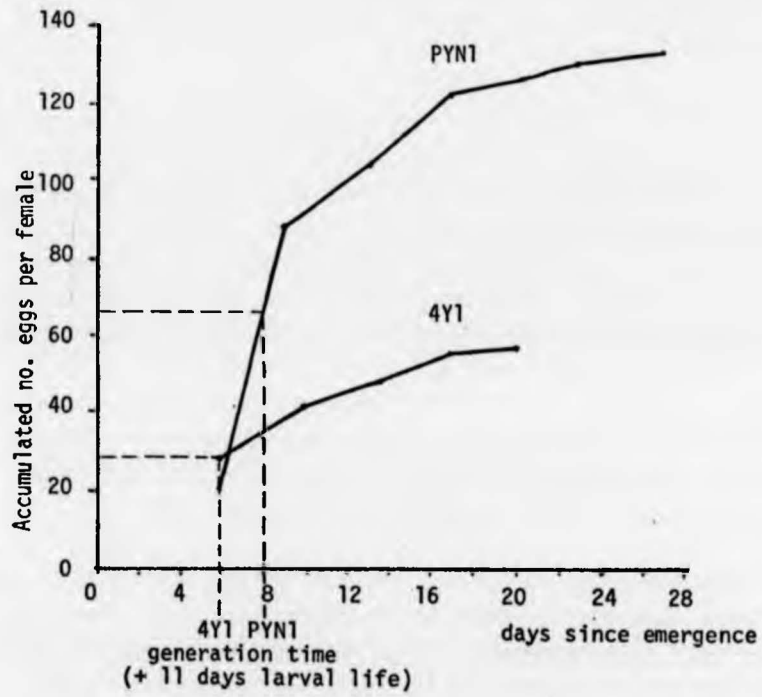
releases) in order to be able to plan the management of the cage population with overlapping generations. For each strain, a 30 cm cube cage containing 50 males and 50 females (newly emerged) was set up. Guinea pigs for blood-feeding and egg bowls for oviposition were provided twice a week. Deaths were counted daily and eggs counted twice a week.

The median adult survival (i.e. time to 50% death) was obtained from graphs showing percentage survival of males and females of each strain with time (Figs. 11 and 12). From Fig. 11 it can be seen that the median adult survival for PYN1 was 12 days for females and 15 days for males. From this it was calculated that introducing 175 newly-emerged adults of each sex per week into the cage would lead to a population size of approximately 300 females and 375 males at steady state. The median adult survival of 4Y1 males was found to be 14 days (Fig. 12). Releases of 525 4Y1 males per week should lead to a final number of 1050 males once steady state is reached; they would be in competition for matings at first only with PYN1 males and later with the offspring of PYN1 X 4Y1 matings also.

Data on egg production of the two strains PYN1 and 4Y1 indicated that, under these laboratory conditions, the females lay their first egg batch on day 6 after emergence (after the first bloodmeal has been taken on day 3) and continue to lay until the end of their lives. Fig. 13 shows the accumulated number of eggs produced by each strain. It can be seen that the egg yield per initial female of 4Y1 (55) is less than half as many as for PYN1 females (131). The female generation time, defined as larval life plus adult life up to the time of laying the median egg, was calculated for each strain from Fig. 13, with larval life taken as 11 days. The generation time was found to be 19 days for PYN1 and 17 days for 4Y1. These values were used to calculate the expected rate of decline of susceptibility in the cage population once releases of 4Y1 into the cage were started.

Once the population of PYN1 had been set up in the 1 m cube cage and had been cycling for several weeks, its egg hatch averaged 78% and survival from first instar to adult was 67% at a density of 300 larvae/2 l of water.

Fig. 13. Egg production by A.gambiae s.s. strains PYN1 and 4Y1.



.s. strains PYN1 and 4Y1.

PYN1

4Y1

20 24 28
days since emergence

4.2.1.2 Progress of the replacement

After the cage population had been cycling for 20 weeks and had reached a stable population size and age distribution, releases of 4Y1 males were started. At first, the level of susceptibility in the cage population was monitored every two weeks. Four- to six-day-old mosquitoes derived from eggs laid by the cage population were infected, concurrently with BEECH control mosquitoes, each batch being fed on the same mouse infected with P. y. nigeriensis as described in the Materials and Methods section. Susceptibility was assessed in the usual two ways:

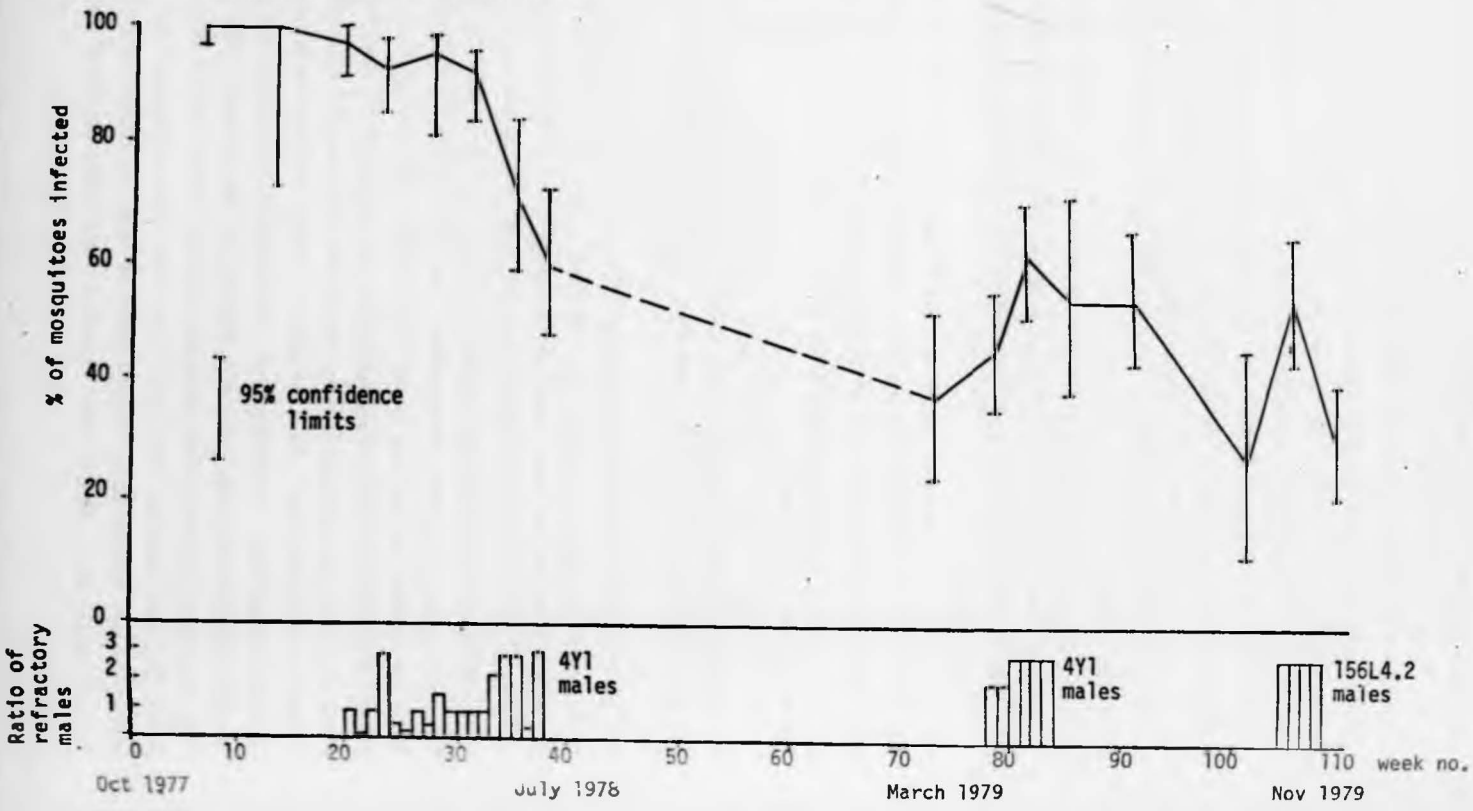
i) the percentage infected (based either on gut and gland preparations, or on guts only if dissections were done before day 8);
ii) the geometric mean of oocyst number per positive mosquito (G.M.(pos)).

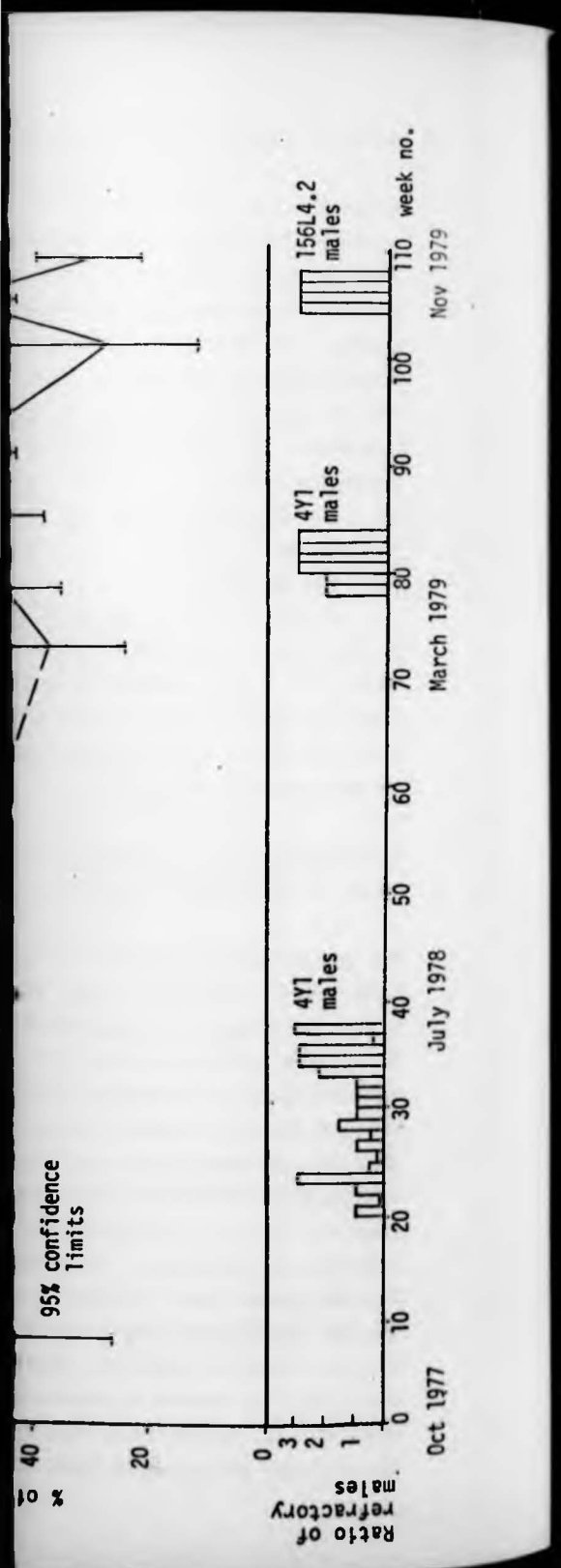
In the early experiments, much variation was found in the intensity of infection in the control mosquitoes, and the G.M.(pos) for samples from the cage population was therefore expressed as a percentage of the G.M. (pos) of the A. stephensi BEECH controls, and called the "standardized G.M.(pos)".

The results of the infections of samples from the cage population are shown in Appendices 2 and 3, together with concurrent control infections.

The percentages infected in samples from the population over the whole experiment are shown in Fig. 14, together with 95% confidence limits. Since the samples of cage population mosquitoes obtained from the two-weekly infections were often small, the values plotted in Fig. 14 were obtained by combining samples in pairs to give monthly values of the percentage infected, except for two cases when it happened that only one infection per month had been done. The confidence limits are not calculated from the standard error of a number of samples, but are obtained from the probabilities predicted for proportions by the binomial distribution. For samples in which the observed percentage in each class (i.e. refractory or susceptible) was greater than 15%, the 95% confidence limit was calculated by the formula $1.96 \times \sqrt{\frac{pq}{n}}$. For the cases in which the observed number in one class was less than 15%, the binomial probabilities have been tabulated and are available in statistical tables such as that in Fisher and Yates (1963). Use of such tables means that the 95% confidence interval is unequally

Fig. 14. Cage replacement experiment: Percentage susceptibility in the cage population and relative numbers of refractory males released.





distributed on either side of the observed value, and avoids the anomaly of the confidence interval extending beyond 0% or 100%.

Fig. 14 also shows the ratios of refractory males released into the cage per week. The ratios given are relative to the number of cage population males replaced per week, not to the standing population of cage males.

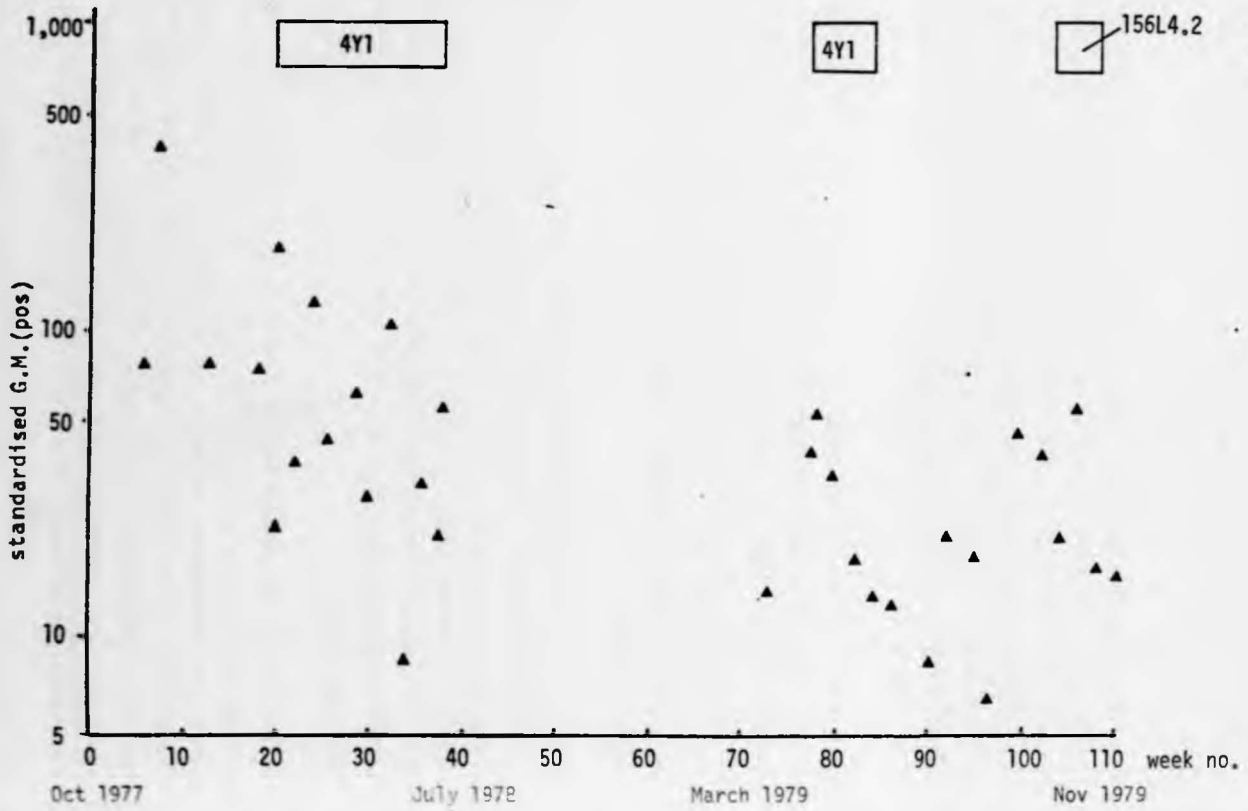
At a time when the susceptibility of 4Y1 was approximately 25%, during February to July 1978 (see Fig. 6), releases were carried out for 18 weeks (weeks 20 to 37). The ratios of 4Y1 released were erratic because of the difficulty of rearing 4Y1. It can be seen from Fig. 14 that after 19 weeks of releases of 4Y1 refractory males, the susceptibility to P. y. nigeriensis in the cage population had declined from 100% to 60 ± (S.E.) 6.3% at week 38.

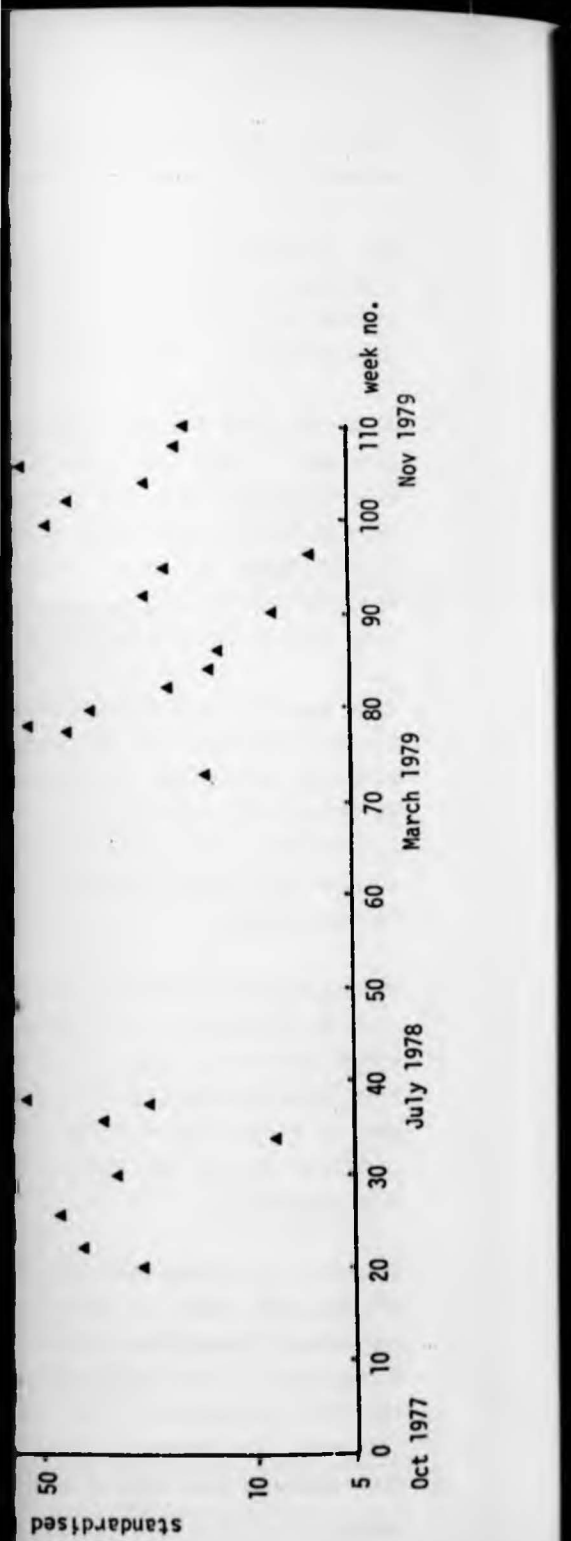
From week 38 (July 1978) until week 73 (March 1979) the susceptibility in the population was not monitored, although males and females were returned to the cage at the rate of 175 of each sex per week as usual. In March 1979 (week 73), the susceptibility of the population was 38.3 ± 7.1% (Fig. 14). The susceptibility had therefore stayed down in the absence of further releases. This important result will be considered further below.

In an attempt to reduce the cage population susceptibility still further, releases of 4Y1 males were carried out for six weeks during weeks 78-83 at a ratio of 2:1 or 3:1 of released males:males emerging from those reared from the cage population. The releases were stopped when it became apparent that the susceptibility of 4Y1 itself had increased to 54% (see Fig. 6) which was approximately the level in the cage population.

Selection for increased refractoriness was immediately started with 4Y1 and with 156L1, as described earlier, to try and create a more refractory strain for release. By generation 4Y5.4 (see Fig. 4), susceptibility had still not decreased in the 4Y line, and therefore the 156L1 derivative 156L4.2 (Fig. 5) was chosen for a third batch of releases. Its susceptibility was 15.2 ± 6.6% in October 1979, when four weeks of releases at a ratio of 3:1 of 156L4.2 males : cage

Fig. 15. Cage replacement experiment: intensity of infection in samples from the cage population.





population males re-cycled were made in weeks 104-107. A decline in susceptibility to a final value of 30.4 ± 4.3% was observed (Fig. 14).

The values for the standardised G.M. (pos) for samples from the cage population are shown in Fig. 15. These have not been pooled to give monthly estimates, since each is standardised to its own control G.M.(pos) for A. stephensi BEECH mosquitoes fed on the same mouse. (see Appendix 3). The intensity of infection showed large fluctuations. Nevertheless, an overall declining trend was observed over weeks 20-38, during releases of 4Y1 with 25% susceptibility. After week 73, the standardised G.M.(pos) seemed to decline after the release of 4Y1 males. This may be due to the fact that although the percentage susceptibility of 4Y1 at that time was no less than that of the cage population, as mentioned above, the G.M.(pos) value of 4Y1 was 10.5, which was considerably lower than the values of 40-55 observed during weeks 77-78 in the cage population (Fig. 15). However, the standardised G.M. (pos) rose again until week 106 after which there is an indication that releases of 156L4.2 males pushed it down again to a final value of 16.9 (Fig. 15).

The results obtained in the cage replacement therefore fall into two distinct phases, separated by an interval of seven months. In the first phase, up to week 38, the results show firstly that the population was established with 100% susceptibility. There was then a continuous period of releases of a refractory strain for 18 weeks, although the relative numbers released varied from week to week. Overall, the average ratio of released:cage males returned was 1:1.14. This was found to be enough to push the susceptibility down to 60%, when the releases had to be terminated. The intensity of infection also showed some evidence of a decline during the first phase of releases.

The second phase of the experiment was more confused. Further releases of 4Y1 had to be abandoned when its susceptibility was found to be no lower than that of the cage population. No strain with lower susceptibility was available until selection had produced strain 156L4.2 with a susceptibility of approximately 15%. Although a short burst of releases at the relatively high ratio of 3:1 showed a promising decline in susceptibility, lack of time necessitated terminating the experiment with the susceptibility level essentially the same as it had been at week 73, at the start of the second phase. The intensity

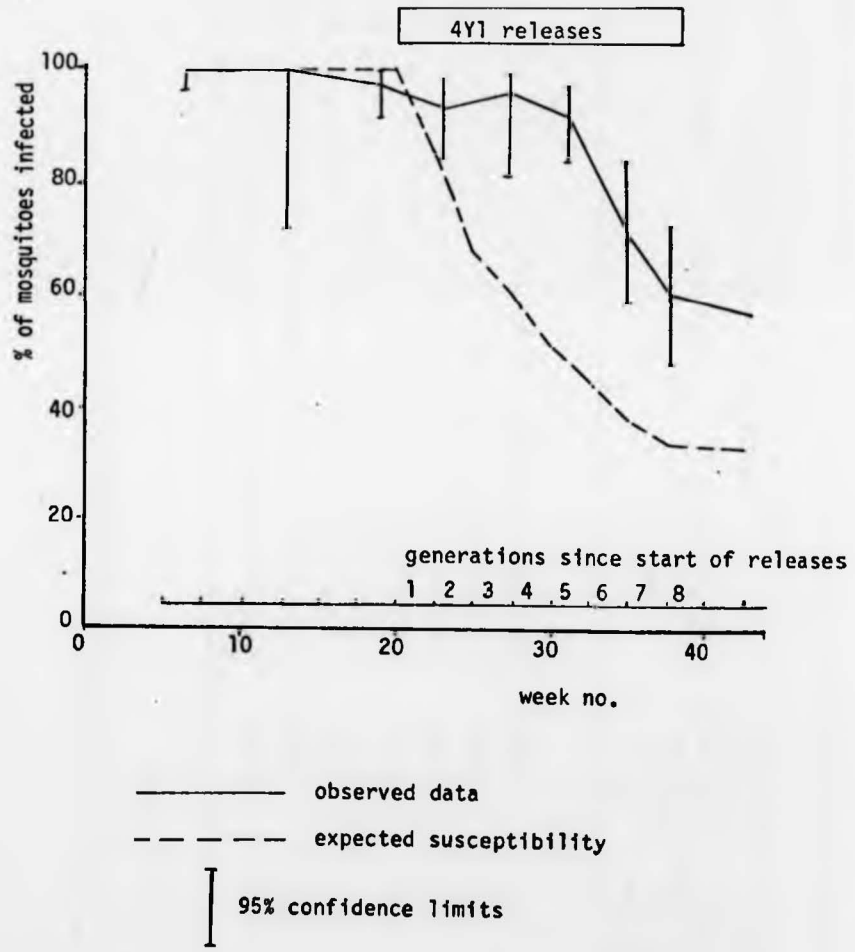
Table 13. Calculation of expected decline in susceptibility in the cage population during the first phase of releases

Generation no.	Average no. 4Y1 males released (r) relative to no. of cage males	Frequency of released males among total males returned $\frac{r}{1+r}$	Contribution to gene frequency by released males	cage males	cage females	Proportion of susceptibility
0	0	0	0	0.5* x 1	0.5* x 1	1.0
1	0.64	0.3902	0.3902 x 0.5 x 0.25	0.6098 x 0.5 x 1	0.5 x 1	0.8537
2	1.30	0.5652	0.5652 x 0.5 x 0.25	0.4348 x 0.5 x 0.8537	0.5 x 0.8537	0.6831
3	0.56	0.3590	0.3590 x 0.5 x 0.25	0.6410 x 0.5 x 0.6831	0.5 x 0.6831	0.6054
4	1.13	0.5305	0.5305 x 0.5 x 0.25	0.4695 x 0.5 x 0.6054	0.5 x 0.6054	0.5111
5	1.0	0.50	0.5 x 0.5 x 0.25	0.5 x 0.5 x 0.5111	0.5 x 0.5111	0.4458
6	2.20	0.6875	0.6875 x 0.5 x 0.25	0.3125 x 0.5 x 0.4458	0.5 x 0.4458	0.3785
7	1.89	0.6540	0.6540 x 0.5 x 0.25	0.3460 x 0.5 x 0.3785	0.5 x 0.3785	0.3365
8	0.38	0.2754	0.2754 x 0.5 x 0.25	0.7246 x 0.5 x 0.3365	0.5 x 0.3365	0.3246

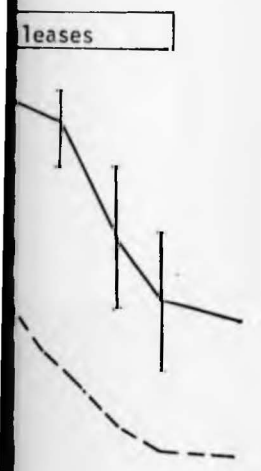
generation time = 2.5 weeks

*the factor 0.5 enters each contribution because each sex contributes 50% of autosomal genes to the next generation

Fig. 16. Cage replacement experiment: observed and expected decline in susceptibility during first period of releases.



observed and expected decline in
eriod of releases.



ns since start of releases
4 5 6 7 8
30 40
week no.

ibility

of infection also showed no net change over the second phase of the experiment.

Attention will therefore be concentrated on the first phase. The expected decline in susceptibility in the cage population was calculated as shown in Table 13 for discrete generations, assuming equal fitness of 4Y1 and PYN1 (the released and target strains). From data previously obtained (Fig. 13) the female median generation time was found to be 19 days for PYN1 and 17 days for 4Y1. In order to be able to calculate the ratios of released males per generation shown in column 2 of Table 13, the generation time was estimated as 2.5 weeks, i.e. 17.5 days, which is intermediate between the two strains. The calculations shown in Table 13 are applicable to refractoriness which is either due to a single gene or to additive polygenes. The cage population males, which, after the first generation of releases, have susceptibility lower than 100%, compete with released 4Y1 males for matings.

The expected decline in susceptibility during the first phase of releases (from the last column in Table 13) is plotted in Fig. 16, along with the observed data. The value plotted is the gene frequency of susceptibility. This is equivalent to the phenotype frequency in cases where there is intermediate dominance. This is a reasonable assumption in the present case, since the expected percentage susceptibility from a cross between strains of 25% and 100% susceptibility would then be 62.5%. The susceptibility of the F_1s from reciprocal crosses between 4Y1 and PYN1 was found to average 68.8% (see Table 7).

Fig. 16 shows that the decline in susceptibility during the first phase of the experiment was slower than predicted by the model which assumes equal fitness of released and 'target' strains. Investigations of the possible reasons for this slow decline were then carried out.

4.2.2 Tests of fitness of the refractory strain

Data obtained at the stage of planning the management of the cage population had indicated that 4Y1 had poor egg yield compared to PYN1 (see Fig. 13). The median adult survival times and the median generation time were also slightly lower for 4Y1 than for PYN1.

Table 14. Mating competition test between strains 4Y1 and PYN1

	Egg batches		% of matings by 4Y1
	Hatched	Unhatched	
(1) Irrad. 4Y1 males + PYN1 males	29	7	19.4
(2) Irrad. PYN1 males + 4Y1 males	9	35	20.5
		Average	20.0

Table 15. Insemination rates for strains 4Y1 and PYN1

Strain	No. inseminated	No. not inseminated	% insemination
4Y1	14	3	82.4
PYN1	27	6	81.8

Table 16. Mating competition test between strains 156L1 and PYN1

	Egg batches		% of matings by 156L1
	Hatched	Unhatched	
(1) Irrad. 156L1 males + PYN1 males	11	7	38.9
(2) " " "	53	10	15.9
		Average	21.9

between strains 4Y1 and PYN1

Egg batches		% of
Hatched	Unhatched	matings by 4Y1
29	7	19.4
9	35	20.5
	Average	20.0

trains 4Y1 and PYN1

No. not inseminated	% insemination
3	82.4
6	81.8

between strains 156L1 and PYN1

Egg batches		% of
Hatched	Unhatched	matings by 156L1
11	7	38.9
53	10	15.9
	Average	21.0

Whilst low egg yield could partly account for poor performance of 4Y1 in reducing the susceptibility in the cage replacement experiment (and incidentally for the difficulty experienced in rearing 4Y1), there are other factors which could be operating, namely mating competitiveness, egg hatchability, larval survival and pupal emergence rate. Such factors are obviously compensated for in rearing 4Y1 males for release, since a known number of adults are released; but once the cage population comprises a mixture of genotypes then factors affecting fitness which were linked to refractoriness could cause selection against refractoriness genotypes.

Mating competition tests were carried out between 4Y1 and PYN1 using radiation sterilisation as a 'marker', with 100 males of each strain and 100 virgin PYN1 females as described in the Materials and Methods section. The results are shown in Table 14. It was found that 4Y1 males have a competitiveness (calculated by Haisch's method (1970)) of 25% compared with PYN1 males. The similar proportions of matings by 4Y1 males obtained in test 1 and test 2 show that the irradiation itself did not affect the mating performance.

4Y1 males are not unconditionally bad at mating, since the insemination rate for each of the two strains was found to be 82% when 50 males and 50 virgin females (all of the same strain and one day old) were placed in a 30 cm cube cage and allowed seven days for mating (Table 15). This demonstrates also that poor insemination cannot account for the difficulty of rearing 4Y1. When the two strains are in competition, however, as they are in the cage replacement experiment, 4Y1 has greatly reduced success at mating, either owing to positive assortative mating by PYN1 females or to slower mating by 4Y1.

156L1 was also tested for its competitiveness compared to PYN1. In both competition tests with 156L1, 100 males were irradiated and competed with PYN1 males of the same age for PYN1 females. The first experiment gave a very poor yield of egg batches. The results are shown in Table 16. When data from the two experiments are combined, 156L1 is found to have a mating competitiveness of 26.6% compared to PYN1, a value very similar to that of 4Y1. This suggests that refractoriness itself may be a cause of lack of fitness, although the result is not conclusive, since 156L1 and 4Y1 were derived in the same series of crosses and similarities between them are to be expected.

Table 17. Egg hatchability and larval survival of strains PYN1, 4Y1 and 156L1 during January-June, 1978.

	PYN1	4Y1	156L1
Egg hatchability (%)	80.1(6,812)	38.7(3,520)	57.8(3,297)
Larval survival, 1st instar to pupa (%)	81.9 (819)		52.0(1,200)
Immature survival, 1st instar to adult (%)	67.3 (400)	43.2 (296)	
Pupal emergence - male (%)	90.5(3,014)	63.4 (243)	
Pupal emergence - female (%)	92.6(2,904)		

( ) = number in sample

Table 18. Larval survival and competitiveness of PYN1 and 4Y1

Competition	Replicate	Normal eye	White eye	χ^2 (1:1 expectation)
PYN1 vs A	(1)	18	36	
	(2)	62	67	
		80	103	3.36 N.S.
4Y1 vs A	(1)	77	79	
	(2)	109	114	
		186	193	0.02 N.S.

Table 19. Relative fitness of 4Y1 compared to PYN1

	PYN1	4Y1	4Y1 relative to PYN1
Mating competitiveness (C)			0.25
Egg yield per female	131	55	0.42
Egg hatchability	80.1%	38.7%	0.48
Larval yield (L)	104.9	21.3	0.203

l survival of strains PYN1, 4Y1 and
1978.

PYN1	4Y1	156L1
80.1(6,812)	38.7(3,520)	57.8(3,297)
81.9 (819)		52.0(1,200)
67.3 (400)	43.2 (296)	
90.5(3,014)	63.4 (243)	
92.6(2,904)		

er in sample

itiveness of PYN1 and 4Y1

mal eye	White eye	χ^2 (1:1 expectation)
18	36	
62	67	
80	103	3.36 N.S.
77	79	
109	114	
186	193	0.02 N.S.

ompared to PYN1

N1	4Y1	4Y1 relative to PYN1
		0.25
31	55	0.42
.1%	38.7%	0.48
04.9	21.3	0.203

Egg hatchability and immature survival rates were also determined for PYN1, 4Y1 and 156L1. Larval survival was measured at a density of 300 first instar larvae per 2 l of water. The results obtained are shown in Table 17. There was a large difference between the egg hatchability of strains PYN1 and 4Y1, and also some difference in the survival through the immature stages of pure cultures of the two strains. It seemed possible that these differences would be accentuated when the two strains were in direct competition. This was tested by assessing the survival of each strain in competition with the white-eyed strain 'A', which was used so that emergents of each strain could be easily identified. For each of the strains PYN1 and 4Y1 two replicate bowls were set up, each containing 150 larvae of PYN1 or 4Y1 and 150 larvae of strain A in 2 l of water. The numbers of normal and white-eyed emergents are shown in Table 18. No significant differences were found between the larval survival of strains PYN1 and 4Y1 in competition with A. It is inferred, therefore, that there would be little difference in the survival of larvae to adults of PYN1 and 4Y1 when in competition with each other.

In summary, there are three respects in which 4Y1 performs badly compared to PYN1. Firstly the egg yield per female (as shown in Fig. 13), secondly the mating competitiveness, and thirdly the egg hatchability. The effect of these factors is shown in Table 19. The combined effect of egg production and egg hatchability (called the 'larval yield') is the product of the two factors, i.e. 0.203, and on this criterion the fitness of 4Y1 is 20.3% of that of PYN1.

4.2.3 Effect of reduced fitness on population replacement

Since the extent to which 4Y1 has reduced fitness compared to PYN1 has been quantified, the effect which this is expected to have on the decline in susceptibility in the cage replacement experiment can be considered.

Since 'larval yield' is a factor which operates after mating, it seems unlikely that anything like the low level obtained for 4Y1 (0.203) would operate in the cage population, firstly because matings between released males and cage population females would result in heterozygotes which might be expected to have 'larval yield' intermediate between 0.203 and 1, and secondly because the heterosis arising from the fact that the matings are between two inbred strains might be expected

Table 20. Method of calculating expected susceptibility at each generation, assuming single gene control of refractoriness, and reduced fitness of refractory type

In this example: Cage population is 100% susceptible.
This is the first generation of releases.
Ratio of refractory males released = 0.64.
All refractory males have competitiveness (C) which is 25% of that of susceptible males.
All refractory males have larval yield (L) which is 50% of that of susceptible males.

	gene frequency of susceptibility amongst:			
	cage susceptible	cage refractory	released refractory	
GEN 1	1.0	0.0	0.64	←Relative numbers returned to cage population
				Divide through by total
	0.6098	0.0	0.3902	←Relative frequency
				Multiply total frequency of refractory types by 0.25 (competitiveness factor)
	0.6098		0.0976	Divide through by total
	0.8620		0.1380	←Relative frequency of males mating
	1.0		0.0	←Relative frequency amongst females
	0.9310		0.0690	←Relative frequency after mating
				Multiply refractory types by 0.5 (larval yield)
	0.9310		0.0345	Divide through by total
	0.9643		0.0357	←Relative frequency amongst larvae and adults of next generation
GEN 2	0.9643		0.0357	1.30
	etc.			

Expected phenotypic susceptibility = 0.9643 + (0.25 x 0.0357) /since refractory stock shows 25% susceptibility/ i.e. 0.9732

Fig. 17. Expected change in susceptibility in the cage population assuming competitiveness of 4Y1 = 0.25 and various values of 'larval yield'.

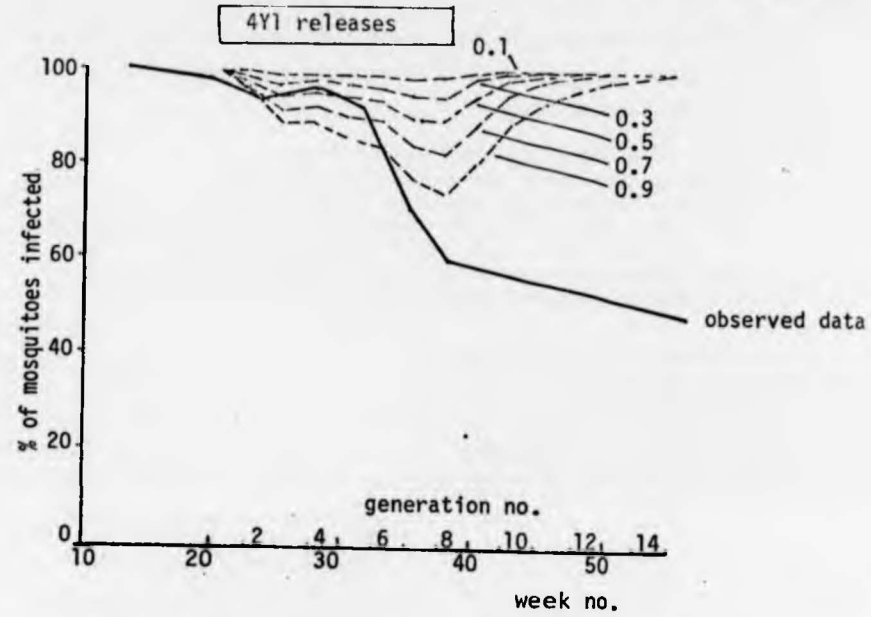
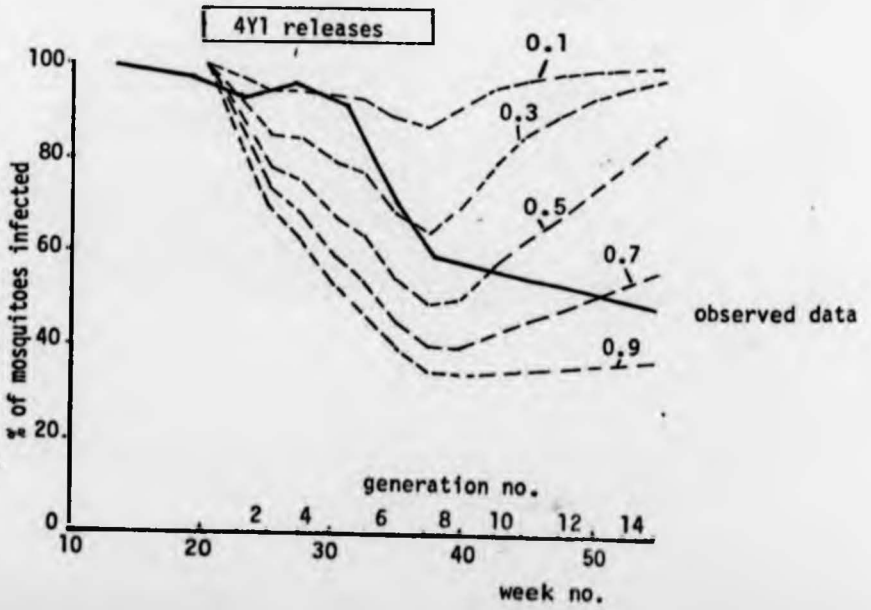


Fig. 18. Expected change in susceptibility in the cage population assuming competitiveness of 4Y1 = 1 and various values of 'larval yield'.



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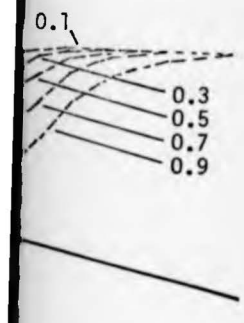

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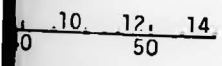

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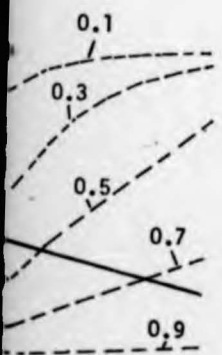

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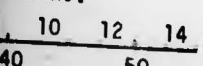


week no.

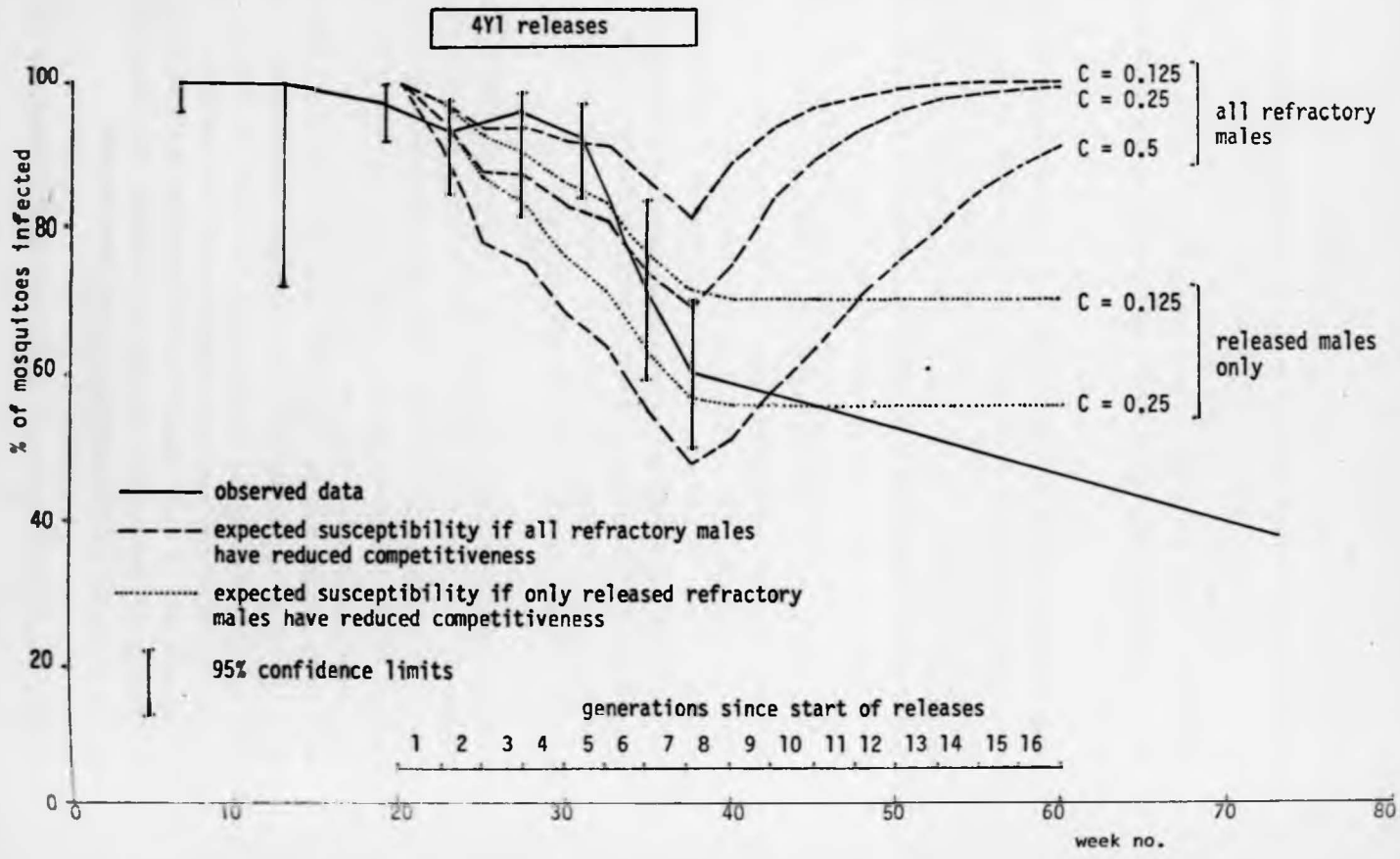
to increase the egg yield and hatchability above that of either of the parent strains. Nevertheless the expected effect of reduced 'larval yield' (L) with values of 0.1 - 0.9 was investigated, as was the expected effect of reduced competitiveness. The calculations were done by programmable calculator as shown in Table 20, with chosen values of C and L inserted. Refractoriness is assumed to be controlled by a single gene which reduces the chance of becoming infected to 25%.

At first the low fitness was assumed to be fully linked to refractoriness. Since the measured value of competitiveness for 4Y1 was 25%, the effect of both C = 0.25 and various values of L was first calculated (Fig. 17). It is clear that poor competitiveness and 'larval yield' cannot both be operating, since in that case susceptibility would not have declined to 60%, nor would it have remained at a low level after cessation of releases. If the effect of low 'larval yield' is considered alone, the graphs shown in Fig. 18 are obtained; as can be seen the value L = 0.1 describes well the early period of releases, but is inappropriate during the later releases and afterwards. It could be that 'larval yield' of refractory types is poor at first but increases as recombination separates refractoriness from the gene(s) responsible for low yield; however, for the reasons described above it seems unlikely that L even as low as 0.203 would operate even during the first few generations.

Expected susceptibilities assuming three different values of competitiveness (C = 0.125, 0.25, 0.5) and full linkage to refractoriness with L = 1 are shown in Fig. 19. The line corresponding to C = 0.25 is the best fit to the observed data during the period of releases, as might be expected since this was the measured value for 4Y1. Once again it is found, though, that if a gene causing poor fitness is fully linked to refractoriness, the expected susceptibility will start to rise immediately releases end, the speed of approach to 100% susceptibility depending on the magnitude of the reduction in fitness. In the case of C = 0.25 (Fig. 19), the expected susceptibility rises to 98% only 20 weeks after releases have ended. The observed data show no increase in susceptibility over that period.

It seems, therefore, that although poor competitiveness of 4Y1 may

Fig. 19. Cage replacement experiment: observed decline in susceptibility, and expected decline given different values of competitiveness of refractory males.



have been the explanation for the slowness of the decline in susceptibility in the replacement experiment, refractoriness and reduced competitiveness were not caused by the same gene or genes. The two factors could be separated by genetic recombination, after which the poor fitness could be selectively eliminated without affecting the frequency of the refractoriness gene(s). When the refractory strain was first released, there was, by chance, or as a result of it having been intensely selected, association between refractoriness and low fitness. The rate at which all the possible genotypes will achieve equilibrium depends on the amount of linkage between the loci. A reasonable approximation to this situation, given that selection for improved fitness and also hybrid vigour are acting, might be that only the released males have poor competitiveness. The refractory portion of the cage population is thus assumed to be fully fit. Calculation of expected susceptibility is done with a minor modification to the method of Table 20, and the graphs obtained assuming C (released males only) = 0.125 and 0.25 are shown in Fig. 19. The value C = 0.125 best describes the rate of decline in susceptibility, whilst C = 0.25 best predicts the final level of susceptibility. After releases end, the level of susceptibility remains stable by this model.

4.2.4 Theoretical study of the introduction of refractoriness genes using hybrid sterility

There was an indication in the work of Al-Mashhadani et al (1980) that a gene affecting susceptibility to P. berghei is situated on the X-chromosome of A. gambiae s.s. Sterility in the A. gambiae complex is mainly controlled by the X-chromosome (Curtis, 1978c). If there were free cross-mating between strains carrying X- and Y-chromosomes of different species origins, it should be possible to replace one X-chromosome by another by releasing sufficient of the latter to constitute a majority.

This replacement was investigated theoretically by considering an isolated area in which there is a population of A. arabiensis only. The refractory strain available for release is A. gambiae s.s. which carries a gene for refractoriness on the X-chromosome. Using this a stock is created which has the X and Y of gambiae s.s. in the background of mixed autosomes, which are assumed to give the released strain full mating competitiveness for the wild strain. Tables 21 and

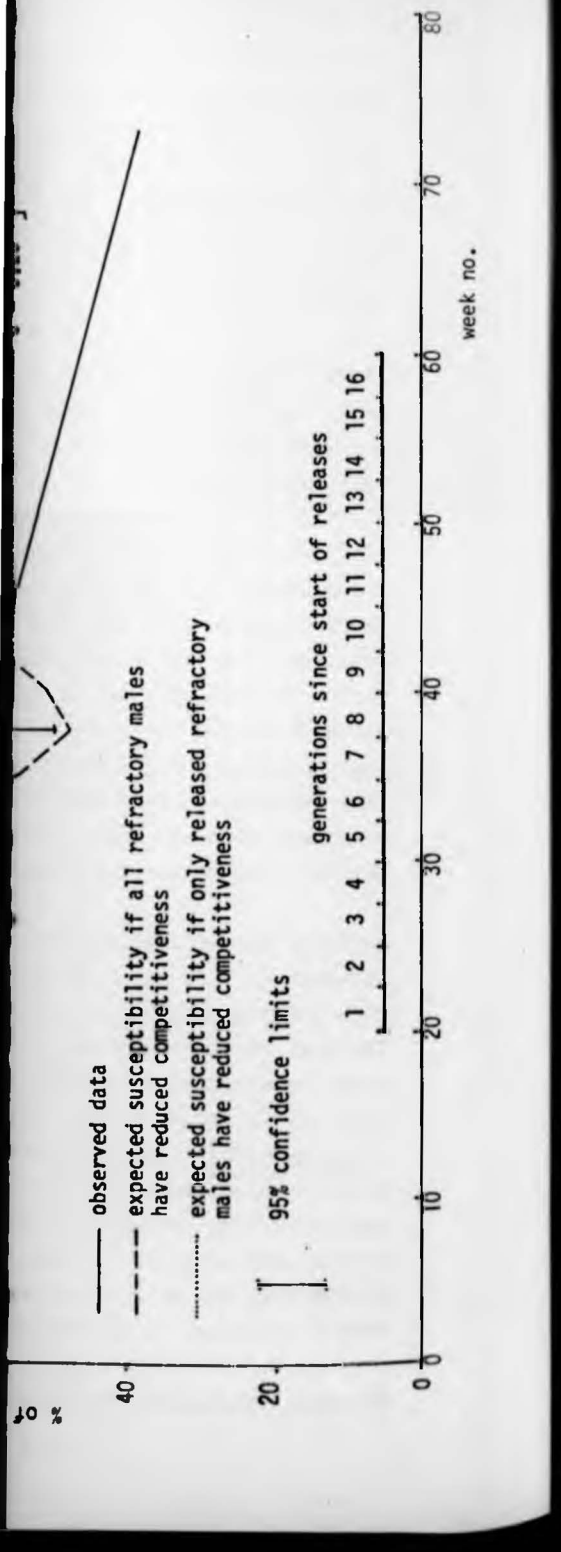


Table 21. Replacement of the sex chromosomes of *A. arabiensis* by one release at a 2:1 ratio of males and females with sex chromosomes of *A. gambiae s.s.* in the background of mixed autosomes.

Generation	Fertile matings: Female	Male	Frequencies after release	Genotype frequency in progeny: Females X_AX_A	X_AX_B X_BX_A	X_BX_B	Males X_AY_A	X_AY_B	X_BY_A	X_BY_B	Frequency of X_B chromosome expected: Females	all males	Fertile males
1	X_AX_A(rel.)	X_AY_A(rel.)	0.67 x 0.67	0.4444			0.4444						
	X_AX_A(rel.)	X_BY_B(wild)	0.67 x 0.33		0.2222			0.2222					
	X_BX_B(wild)	X_AY_A(rel.)	0.33 x 0.67		0.2222				0.2222				
	X_BX_B(wild)	X_BY_B(wild)	0.33 x 0.33			0.1111				0.1111			
	At end of gen. 1: Frequency:			0.4444	0.4444	0.1111	0.4444	0.2222 (sterile)	0.2222 (sterile)	0.1111	0.3333	0.3333	0.2
2	X_AX_A	x X_AY_A		0.1975			0.1975						
	"	x X_BY_B			0.0494			0.0494					
	X_AX_B	x X_AY_A		0.0988	0.0988		0.0988		0.0988				
	"	x X_BY_B			0.0247	0.0247		0.0247		0.0247			
	X_BX_B	x X_AY_A			0.0494				0.0494				
	"	x X_BY_B				0.0123				0.0123			
	At end of gen. 2: Relative nos.			0.2963	0.2223	0.0370	0.2963	0.0741	0.1482	0.0370			
	Frequency:			0.5333	0.4001	0.0666	0.5333	0.1334 (sterile)	0.2667 (sterile)	0.0666	0.2666	0.3333	0.1110
3	X_AX_A	x X_AY_A		0.2844			0.2844						
	"	x X_BY_B			0.0355			0.0355					
	X_AX_B	x X_AY_A		0.1067	0.1067		0.1067		0.1067				
	"	x X_BY_B			0.0133	0.0133		0.0133		0.0133			
	X_BX_B	x X_AY_A			0.0355				0.0355				
	"	x X_BY_B				0.0044				0.0044			
	At end of gen. 3: Relative nos.			0.3911	0.1910	0.0177	0.3911	0.0488	0.1422	0.0177			
	Frequency:			0.6521	0.3184	0.0295	0.6521	0.0814 (Sterile)	0.2371 (sterile)	0.0295	0.1887	0.2666	0.0433

X_AY_A = sex chromosomes of *A. arabiensis*

X_BY_B = " " " *A. gambiae s.s.*

22 show the effect of two release regimes on the frequency of the wild type X-chromosomes. The wild population females are represented by X_BX_B, whilst the released males are X_AY_A. After one release the autosomal composition of the wild population will be mixed, but only the frequencies of the sex chromosome types are shown in the Tables.

Table 21 shows the effect of only one release of both males and females carrying X- and Y-chromosomes of *gambiae s.s.* at a ratio of 2:1 with the wild population. One generation after the release, the frequency of X_BX_B females is reduced to 0.11, but the frequency of the X_B chromosome is still at the level created by the releases, i.e. 0.33. Subsequently selection against X_B begins because it is the minority type and hence proportionately more affected by the fact that some of the males are sterile. After the third generation, the frequency of homozygous X_BX_B females is down to 0.03, the frequency of X_B in females is down to 0.19 and continued decline in these proportions is to be expected.

Although the released females would be refractory, there may be objections to their use and so a scheme involving release of males only has been considered (Table 22). In this case, two releases at a 4:1 ratio of released:wild males have been assumed, and it can be seen that with this regime the frequency of X_BX_B females is down to approximately 0.02 after three generations. Again the decline in frequency continues after releases have ended, and will eventually lead to complete eradication of the *arabiensis* X-chromosome, because of the negative heterosis inherent in the hybrid sterility.

This simple model assumes that X-Y interaction is the only cause of sterility in hybrids (so that all males with X from one species and Y from another are sterile), that the sterility is 100%, and that there is random mating amongst all the different genotypes. The validity of these assumptions will be considered in the Discussion. The model serves to illustrate the principle that theoretically one X-chromosome can be replaced by another using hybrid sterility.

Table 22. Replacement of the sex chromosomes of *A. arabiensis* by two releases at a 4:1 ratio of males with the sex chromosomes of *A. gambiae s.s.* in the background of mixed autosomes

	Genotype Frequencies							Frequency of X_B chromosome amongst:		
	Females			Males						
	X_AX_A	X_AX_B X_BX_A	X_BX_B	X_AY_A	X_AY_B (sterile)	X_BY_A (sterile)	X_BY_B	Females	All males	Fertile males
After 1st release			1.0	0.8			0.2	1.0	0.2	0.2
After gen.1 and 2nd release		0.8	0.2	0.8		0.16	0.04	0.6	0.2	0.0476
After gen. 2	0.3810	0.5905	0.0286	0.3810	0.0190	0.5714	.0.0286	0.3238	0.6	0.0698
After gen. 3	0.6292	0.3484	0.0225	0.6292	0.0471	0.3013	0.0225	0.1967	0.3238	0.0345

Calculated as shown in Table 21

X_A, Y_A = sex chromosomes of *A. gambiae s.s.*

X_B, Y_B = " " 2 *A. arabiensis*

After gen.								
3	0.6292	0.3484	0.0225	0.6292	0.0471	0.3013	0.0225	0.1967	0.3238	0.0345

Calculated as shown in Table 21

X_A, Y_A = sex chromosomes of *A. gambiae s.s.*

X_B, Y_B = " " 2 *A. arabiensis*

4.3 EXPERIMENTS WITH *P. falciparum*

The aims of the experiments at the MRC Laboratories, The Gambia, were 1) to select a strain of *A. gambiae s.s.* refractory to *P. falciparum*; 2) to test the susceptibility to *P. falciparum* of the strains of *A. gambiae s.s.* which are refractory or susceptible to *P. y. nigeriensis*.

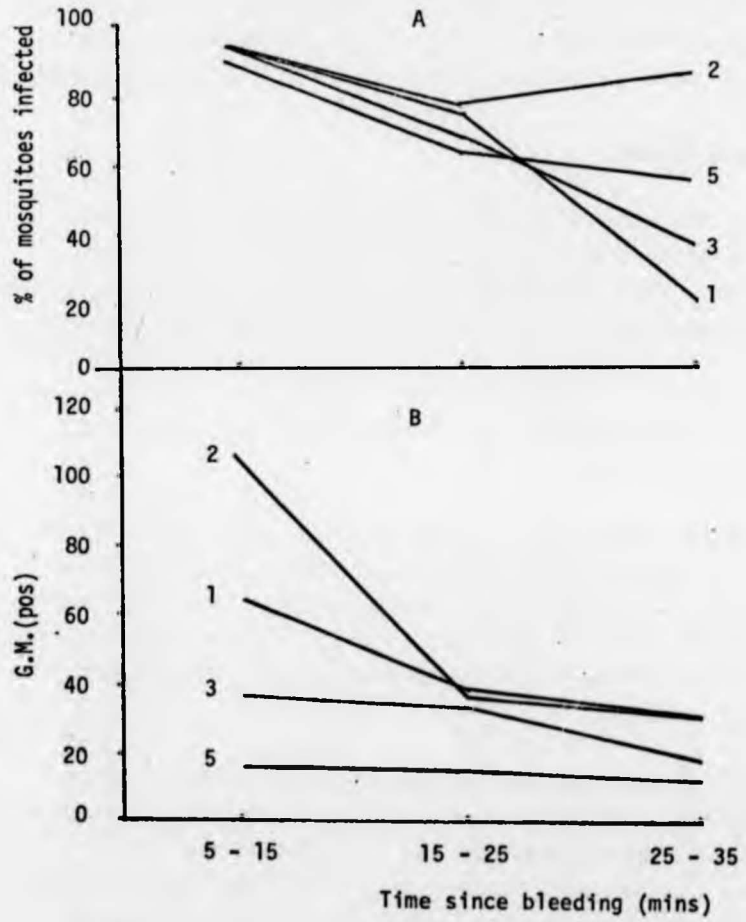
4.3.1 Membrane feeding

In contrast to the work by Al-Mashhadani (1976), feeds could not be carried out directly on patients, since this is now ruled out on ethical grounds. Blood drawn from the patients was therefore presented to the mosquitoes through artificial membranes. The first task was to assess how reliable membrane feeding was for infecting mosquitoes. It was very important to eliminate 'false negatives', i.e. mosquitoes which failed to become infected for reasons other than genetically determined refractoriness.

Initially feeding times of up to 50 minutes were used, but this was reduced to 20-25 minutes fairly early on in the work when it became apparent that the length of time available for feeding was important in determining the level of infection obtained. It was later reduced even further to 10 minutes (i.e. 15 minutes in total from when the blood left the patient).

Two experiments on the change of infectivity of blood in a feeder during a 35-minute period were carried out by exposing fresh batches of mosquitoes of identical age for three consecutive ten-minute periods to the blood in each feeder. In the first experiment, blood from a patient with 1,186 gametocytes/mm^3 was divided between one large and two small feeders (numbers 1, 2, and 3 in Figs. 20A and B). The remaining blood was introduced into another small feeder (Number 4) on which mosquitoes were not fed, and then at six minutes after bleeding blood films were made from blood left in the container. Thin films were made at intervals from blood withdrawn by syringe through the membrane of feeder number 4. In the second experiment the blood obtained from a patient with 343 gametocytes/mm^3 was only sufficient for one small feeder (number 5 in Figs. 20A and B). In both experiments blood had been placed in the feeders three to four

Fig. 20. Infectivity of P.falciparum - infected blood to A.gambiae s.s. at time intervals in membrane feeders.
A : percentage of mosquitoes infected.
B : intensity of infection.



Feeder	Expt.	Size	Nos. dissected 5-15 min	15-25 min	25-35 min
1	1	large	53	46	26
2	1	small	40	20	26
3	1	small	28	30	20
5	2	small	38	16	11

- infected blood to A.gambiae s.s.
e feeders.
infected.

- 25 25 - 35

ince bleeding (mins)

Nos. dissected		
5 min	15-25 min	25-35 min
53	46	26
40	20	26
28	30	20
38	16	11

minutes after bleeding, but mosquito feeding commenced at five minutes after bleeding.

In three feeders out of four there was an apparent decline in infectivity of the blood over a 35-minute period. In experiment 1, (Fig. 20A) each feeder infected similar numbers of mosquitoes in the first ten-minute period (heterogeneity $\chi^2_2 = 0.0003$, $P > 0.1$) and in the second ten-minute period ($\chi^2_2 = 0.17$, $P > 0.1$). This was not the case in the third period when the numbers infected by each feeder showed considerable heterogeneity ($\chi^2_2 = 12.65$, $P < 0.005$), suggesting that different feeders provide environments of differing efficiency for maintaining the infectivity of the blood. Although the patient in experiment 2 had a much lower gametocytaemia, the percentage of mosquitoes infected in the first ten minutes was very similar to that in experiment 1. Because of the lack of significant heterogeneity between the samples in each of the first two time periods, the data from the four feeders were pooled and the decline in infectivity from the first period to the second was found to be statistically significant ($\chi^2_1 = 21.13$, $P < 0.001$). In the third time period the feeders should be considered separately. Comparing the numbers infected in the third period with those in the second gave the following results: Feeder 1: $\chi^2_1 = 17.98$, $P < 0.001$; Feeder 2: $\chi^2_1 = 0.19$, $P > 0.1$; Feeder 3: $\chi^2_1 = 3.73$, $0.1 > P > 0.05$; Feeder 5: $\chi^2_1 = 0.0002$, $P > 0.1$. Thus in feeder 1 there was a statistically significant further decline in infectivity from the second to the third period. In feeder 3 the decline bordered on statistical significance. In the other two feeders (2 and 5) there was no significant difference between the percentages infected in the second and third periods.

Fig. 20B shows that the geometric mean of oocyst number per positive mosquito varied widely between feeders even when the blood was from the same patient. However an overall decline in oocyst number was apparent over the 35 minute period in all the feeders used in the two experiments. Figs. 20A and B show that for maximum infections to be obtained, it was necessary for mosquito feeding to be completed within 15 minutes or less of the blood leaving the patient.

Blood films were made at intervals from feeder 4 to investigate whether gametocyte activation was prevented in a membrane feeder.

Plate 1. Activated female P. falciparum gametocyte

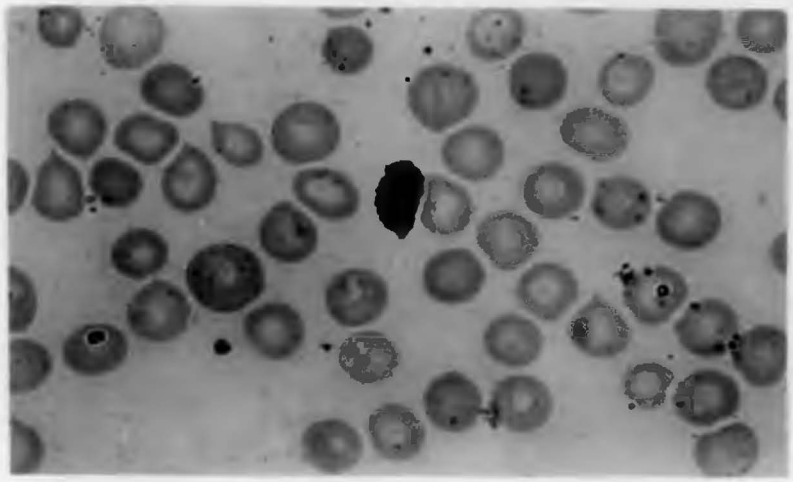


Plate 2. Activated and unactivated P. falciparum gametocytes

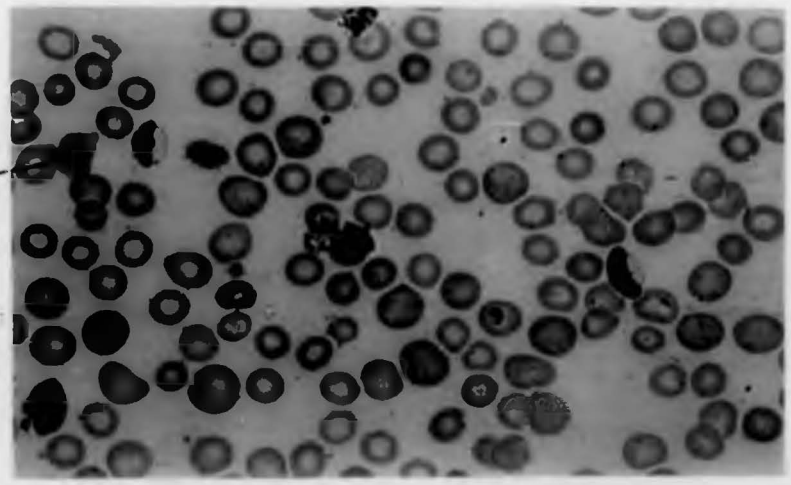


Plate 1. Activated female P. falciparum gametocyte

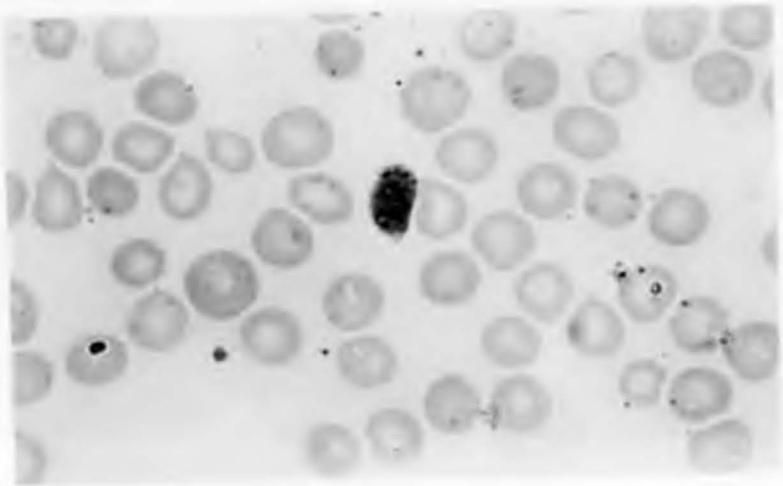


Plate 2. Activated and unactivated P. falciparum gametocytes

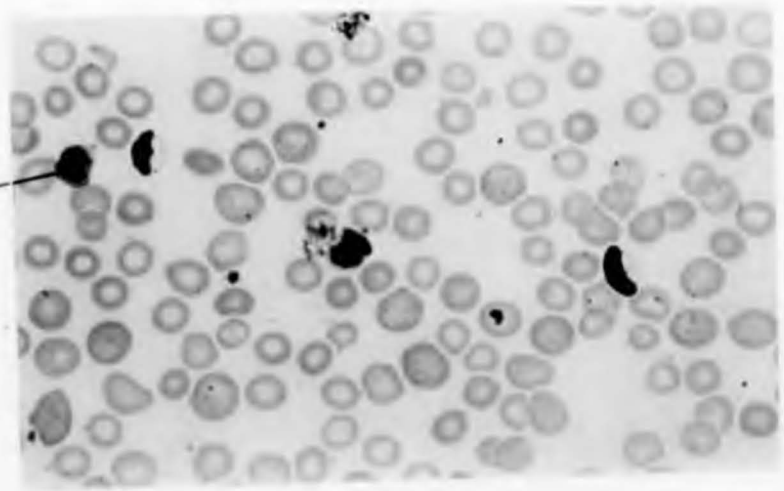


Plate 1. Activated female P. falciparum gametocyte

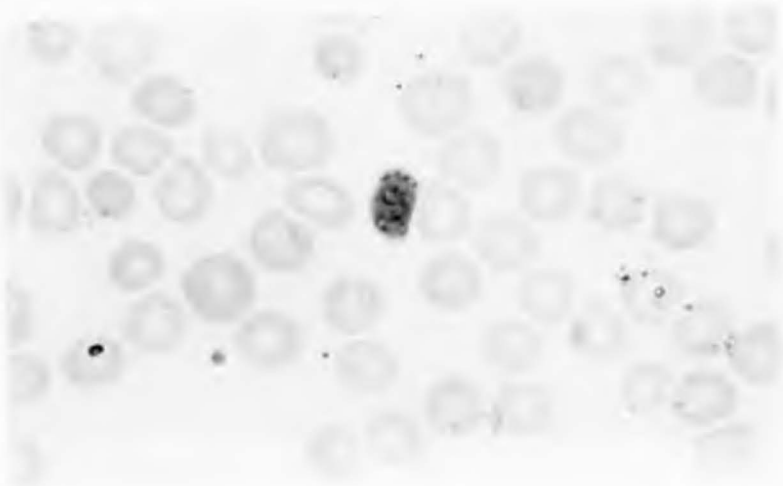


Plate 2. Activated and unactivated P. falciparum gametocytes

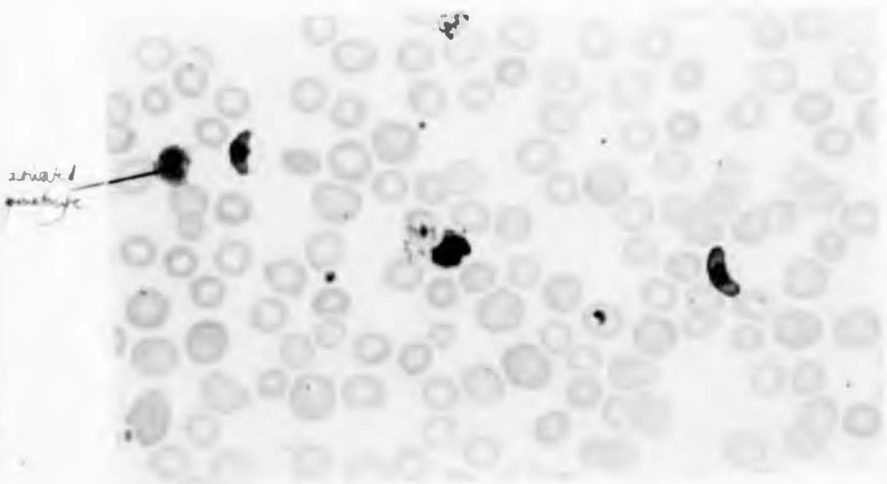


Table 23. P. falciparum gametocytes in blood films taken at time intervals from a membrane feeder

Time from bleeding (mins)	Unactivated gametocytes/10^5 red cells	Sex ratio males: females	% activated female gametocytes
15	66	1:7.9	3
25	67	1:6.4	7
35	68	1:8.4	7
45	56	1:6.6	12
55	36	1:11.3	20

Table 24. Infectivity of P. falciparum infected blood to mosquitoes after suspension in S.A. Medium

	FEED NO. 41				FEED NO. 59			
	No. diss.	% infected	S.N.D.	G.M. (pos)	No. diss.	% infected	S.N.D.	G.M. (pos)
Control (immediate feed)	69	73.9	6.3***	32.4	49	98.0	4.2***	212.7
S.A. medium then F.C.S.	35	8.6		4.0	45	64.4		9.8
Time in S.A. medium	38 mins				73 mins			

*** P < 0.001

Table 25. Comparison between large and small feeders for feeding times of 20 minutes

Feed no.	Feeder size	No. dissected	% infected	S.N.D.	G.M.(pos)
37	Large	99	83.8	1.55 N.S.	51.5
	Small	118	83.1		53.4
48	Large	30	90.0	0.27 N.S.	48.7
	Small	50	88.0		28.5

N.S. Not significant

od films taken at time intervals

Sex ratio males: females	% activated female gametocytes
1:7.9	3
1:6.4	7
1:8.4	7
1:6.6	12
1:11.3	20

ected blood to mosquitoes after

FEED NO. 59

M. os)	No. diss.	% infected	S.N.D.	G.M. (pos)
.4	49	98.0	4.2^{***}	212.7
.0	45	64.4		9.8
		73 mins		

< 0.001

ll feeders for feeding times

% infected	S.N.D.	G.M.(pos)
83.8 83.1	1.55 N.S.	51.5 53.4
90.0 88.0	0.27 N.S.	48.7 28.5

Activation of gametocytes (defined by Sinden et al, 1976, and Sinden & Smalley, 1976) involves rounding in shape and escape from the erythrocyte. An activated female P. falciparum gametocyte is shown in Plate 1 whilst Plate 2 shows male and female unactivated gametocytes, as well as an activated gametocyte.

Examination of thin films made from blood in feeder number 4 gave the results shown in Table 23. During the first 35 minutes there was little change in the number of crescents or the sex ratio, and only a small increase in the percentage of activated female gametocytes. From 35 to 55 minutes after bleeding there was a decline in the number of crescents and a corresponding rise in the percentage of activated females. The sex ratio among unactivated gametocytes shifted in favour of females towards the end of this period, although too few activated male gametocytes were found in the blood films to enable their percentages to be accurately determined. Thin films made from blood taken at the same time which had remained at 27-29°C until six minutes after bleeding showed that 23% of the female gametocytes had activated.

To obviate the need for rapid transfer of blood from patient to insectary, attempts were made to buffer the blood by suspension in S.A. (suspended animation) medium (Nijhout & Carter, 1978). To recover the blood, the red blood cells were washed in fresh S.A. medium, resuspended in bicarbonate-free serum (BFS) or foetal calf serum (FCS) and then immediately introduced into a small feeder and offered to mosquitoes. Mosquitoes were reluctant to feed on the BFS, and therefore no results were obtained when using this resuspension medium.

The results obtained from the immediate control feed and from the blood resuspended in FCS are shown in Table 24. In the first experiment (Feed no. 41), little infectivity was retained, whilst in the second (No. 59) the percentage infected remained relatively high, despite the fact that the blood was kept longer in the S.A. medium than in the first case. The intensity of infection assessed by the G.M.(pos) dropped considerably in both experiments. In neither case was enough blood obtained for a second control, of blood left untouched for the same length of time, to be carried out.

It was noted that on some occasions, different feeders containing blood from the same patient had varying infectivity to batches of mosquitoes. It seemed possible that the different sized feeders might vary in their ability to preserve the infectivity of gametocytes, perhaps by maintaining a more uniform temperature of the blood.

Table 26. P. falciparum gametocyte carriers classified by age and gametocytaemia

Age (years)	Gametocytes/mm^3				Total	%
	< 100	101-300	301-1,000	>1,000		
<1	0	5	5	2	12	25.5
1-4	6	7	10	4	27	57.4
5-14	2	2	1	1	6	12.8
>15	1	0	0	1	2	4.3
Total	9	14	16	8	47	100.0

Table 27. Proportion of infective P. falciparum gametocyte carriers by age and gametocytaemia*

Age (years)	Gametocytes/mm^3				Total	% infective in each age group
	<100	101-300	301-1,000	>1,000		
<1		2/5	3/5	1/2	6/12	50.0
1-4	2/6	3/7	8/10	4/4	17/27	63.0
5-14	0/2	1/2	1/1	1/1	3/6	50.0
>15	0/1			1/1	1/2	50.0
Total	2/9	6/14	12/16	7/8	27/47	
% infective for each gametocytaemia	22.2	42.8	75.0	87.5		

* "infective" means that at least one mosquito became infected out of a sample of at least 10 dissected

rriers classified by age and

rtes/mm^3 1,000	>1,000	Total	%
5	2	12	25.5
0	4	27	57.4
1	1	6	12.8
0	1	2	4.3
5	8	47	100.0

falciparum gametocyte carriers by

^{3} ,000	>1,000	Total	% infective in each age group
5	1/2	6/12	50.0
10	4/4	17/27	63.0
	1/1	3/6	50.0
	1/1	1/2	50.0
16	7/8	27/47	
0	87.5		

at least one mosquito became infected
east 10 dissected

However in two experiments in which this was tested directly, no significant difference was observed in the percentage infected from the large and small feeders (Table 25). In one of the experiments, though (Feed no. 48), a lower G.M.(pos) was obtained from the small feeder. This confirms the observation made from Fig. 20B that the G.M.(pos) cannot be regarded as a reliable measure of the infectivity of P. falciparum-infected blood when studied by membrane feeding. The percentage infected, on the other hand, was usually very similar between replicate feeders for feeds of 20 minutes or less (Fig. 20A and Table 25).

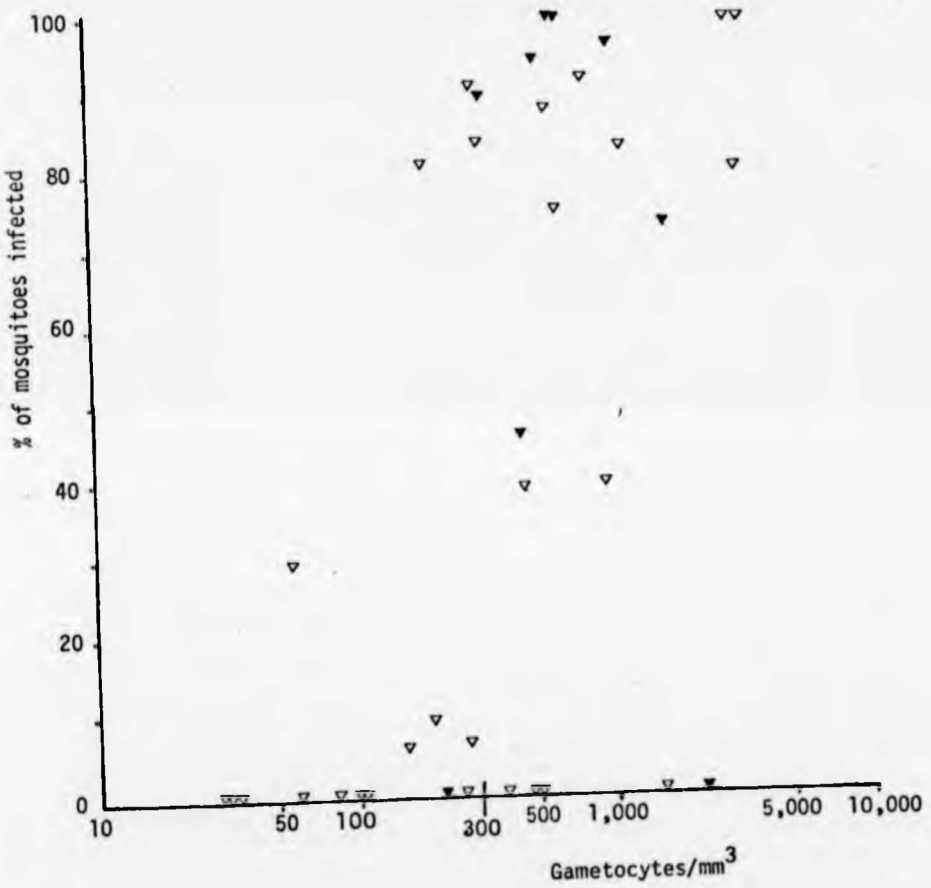
4.3.2 Infectivity of P. falciparum gametocyte carriers

Patients with as high gametocytaemia as possible were selected. However, gametocytaemias had to be estimated rapidly and a wide range were therefore obtained. No account was taken of age in selecting patients, although most of the high gametocytaemias were in children under five, as can be seen from Table 26.

Table 27 shows the proportion of infective carriers in each age-group and level of gametocytaemia. There is no evidence that the proportion of infective carriers in each age-group differs, when all gametocytaemias are included (Heterogeneity $\chi^2_3 = 0.79$, N.S.). However Table 27 shows that, over all age-groups, the proportion of infective carriers increases with gametocytaemia, from 22.2% of those with less than 100 gametocytes/mm^3 to 87.5% of those with over 1,000 gametocytes/mm^3 ($\chi^2_3 = 10.76$, $P < 0.05$).

In Table 27 there is no information about the level of infection produced by each carrier, since 'infective' is defined only as the ability to infect one mosquito out of a batch of at least ten dissected. Most batches provided between 20 and 60 mosquitoes for dissection. The percentage of mosquitoes infected by each carrier is shown in Fig. 21 . The data in Fig. 21 have been classed into two groups according to length of feed, which was shown to be important (Fig. 20). It can be seen that there was a tendency for the percentage of infected mosquitoes to increase at higher gametocyte counts, although there were some exceptionally low infections produced from high carriers. Few of the blood samples infected 100% of fed mosquitoes, but 21 out

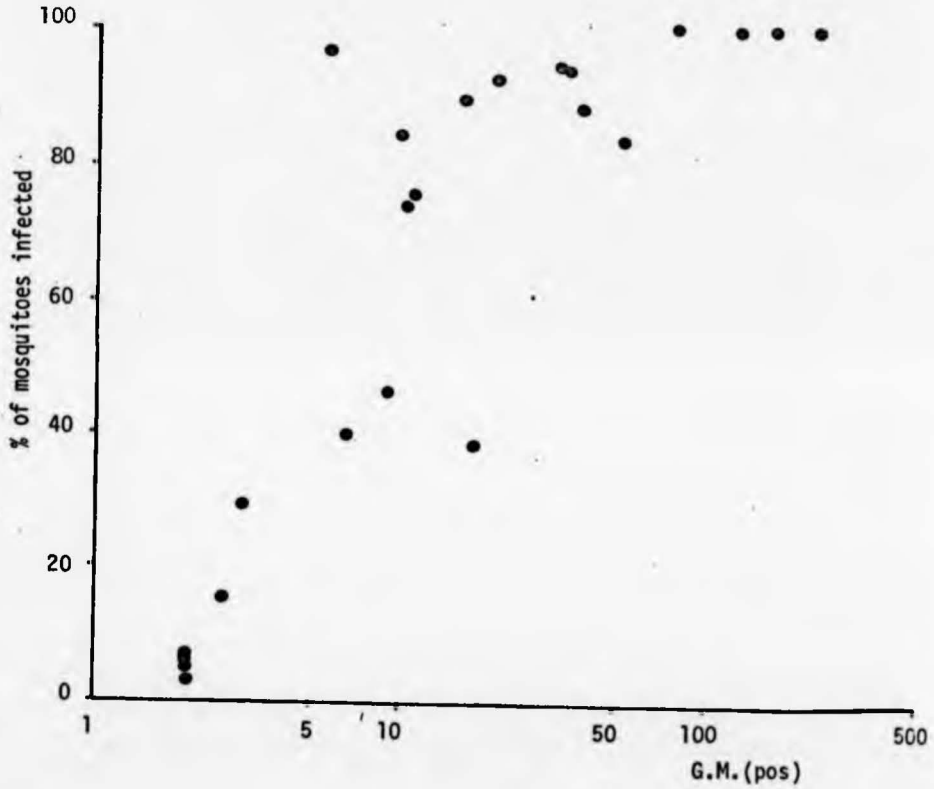
Fig. 21. Percentage of mosquitoes infected in batches of A.gambiae s.s. fed on P.falciparum-infected blood.



▽ 20 - 40 minutes

▼ 10 - 15 minutes

Fig. 22. Relationship between percentage of mosquitoes infected and intensity of infection in batches of A.gambiae s.s. fed on P.falciparum - infected blood.



of 31 blood samples with more than 300 gametocytes/mm^3 infected more than 70% of the mosquitoes. Only one out of 14 blood samples with less than 300 gametocytes/mm^3 infected 70% or more of mosquitoes. The difference between the numbers of high infections produced from the high ($\triangleright$ 300) and low ($\triangleleft$300) gametocytaemias was highly significant ($\chi^2_1 = 11.9$, $P \triangleleft 0.001$).

In Fig. 22 is shown the relationship between the percentage of mosquitoes infected and the intensity of infection, measured by the geometric mean in infected mosquitoes (G.M.(pos)). This shows clearly that in batches of mosquitoes with a high percentage infected, the intensity of infection was also high. Both Figs. 21 and 22 show that the proportion of mosquitoes infected tends towards 100% as the gametocytaemia and the average intensity of infection increase. This suggests that few or none of the FAJARA strain are genetically refractory to infection.

4.3.3 Selection for refractoriness to P. falciparum in A. gambiae s.s.

Two selection lines were set up from the FAJARA strain. The first (LOW 1) was derived from the eggs of three females, whilst the second line (LOW 2) was started from the eggs of four females which had a relatively low infection. In subsequent generations, eggs from those females with low or negative oocyst counts were reared to form the next generation. One or more batches of mosquitoes at each generation were given bloodmeals, because of the difficulty of knowing in advance which patients were going to be infective. Selection was not possible at each generation, because of non-infectivity of blood donors or lack of sufficient egg-batches. It was impossible to use a standard criterion by which females were selected because of variability in the intensity of the infection in a particular experiment and the number of egg batches obtained. At each generation control feedings on the original FAJARA strain were carried out concurrently with feedings on the selected lines.

Table 28 shows the susceptibility of the line LOW 1 over seven generations compared with the control. The results for the earlier infections (especially before feed 26) must be regarded as less reliable than the more recent experiments, since at the start of the work the feeding time used was too long. Only three selections on LOW 1 could

Table 28. Susceptibility to P. falciparum of the line LOW 1 compared to the control FAJARA

Gen. no.	Feed no.	CONTROL			LOW 1			Selection
		No. diss.	% inf.	G.M. (pos)*	No. diss.	% inf.	G.M. (pos)*	
0	3	24	81.8		24	81.8		Yes → gen.1
1	9	13	0	0	3	0		No → gen.2
2	14	31	0	0	5	0	0	No → gen.3
3	25	70	84.3	9.7	10	0	0	No → gen.4
	27	15	0	0	7	0	0	No
4	31	44	88.6	37.4	31	83.9	43.5	Yes → gen.5
	33	16	0	0	16	6.3	3.0	No
	34	49	38.8	16.8	13[a]	38.5	12.4	No
	34	"	"	"	42[b]	92.9	45.2	No
	35	20	75.0	10.5	11	81.8	10.0	No
5	44	30	0	0	8	0	0	No
	45	17	5.9	2	2	50.0	8.0	No
	46	50	0	0	5	60.0	58.5	No
	47	44	0	0	1	0	0	No → gen.6
	48	50	56.0	17.5	4	75.0	20.9	No
	50	17	88.2	6.3	6	83.3	7.8	No
	56	30	73.3	10.0	2	0	0	No
6	60	26	100	163.2	7	85.7	173.5	No → gen.7
	63	13	92.3	33.2	6	66.7	21.5	No
7	65	50	94.0	33.9	27	88.9	12.6	Yes → gen.8

* geometric mean of oocysts per mosquito positive for infection

a using small feeder

b using large feeder

Table 29. Susceptibility of line LOW 2 compared to the control FAJARA

Gen. no.	Feed no.	CONTROL			LOW 2			Selection
		No. diss.	% inf.	G.M. (pos)*	No. diss.	% inf.	G.M. (pos)*	
0	5	30	93.3		30	93.3		Yes → gen.1
1	13	15	6.7	2	0			No → gen.2
2	21	48	0		4	0	0	No
	22	6	50.0	6.3	21	85.7	9.4	Yes → gen.3
	23	5	0	0	12	0	0	No
	26	51	0	0	16	12.5	3.5	No
3	30	29	0	0	8	0		No
	32	53	0	0	3	0	0	No → gen.4
	35	20	75.0	10.5	2	100	45.8	No
4	42	2	50.0	4.0	2	100	3.0	No
	43	30	90.0	48.7	8	87.5	34.6	No
	48	50	56.0	17.5	33	69.7	23.2	Yes → gen.5
	50	17	88.2	6.3	1	0	0	No
5	61	28	0	0	2	0	0	No → gen.6
6	65	50	94.0	33.9	8	62.5	5.2	Yes → gen.7

* geometric mean of oocysts per mosquito positive for infection

2 compared to the control FAJARA

L O W 2			Selection
No. diss.	% inf.	G.M. (pos)*	
30	93.3		Yes ⟶ gen.1
0			No ⟶ gen.2
4	0	0	No
21	85.7	9.4	Yes ⟶ gen.3
12	0	0	No
16	12.5	3.5	No
8	0		No
3	0	0	No ⟶ gen.4
2	100	45.8	No
2	100	3.0	No
8	87.5	34.6	No
33	69.7	23.2	Yes ⟶ gen.5
1	0	0	No
2	0	0	No ⟶ gen.6
8	62.5	5.2	Yes ⟶ gen.7

ito positive for infection

be carried out and it is therefore not surprising that by generation 7 no difference in the percentage susceptible in the LOW 1 and FAJARA strains was observed ($\chi^2_1 = 0.24$, N.S.).

From Table 28, two of the difficulties involved in the work are apparent. Firstly, at Feed 34 can be seen the difference sometimes obtained between replicate cages, in this case of LOW 1 gen. 4, fed on different feeders. Secondly, the problems of trying to inbreed families from single females is apparent at generation 5, in which each sample represents a family reared from single females of LOW 1 gen. 4. It can be seen that the number which fed in each family was very small, and the offspring of all the females had to be pooled for LOW 1 gen. 6.

Table 29 shows the susceptibility of the LOW 2 line over six generations, during four of which selection for low susceptibility was carried out. At generation 6, the percentage infected in the LOW 2 line was significantly lower than in the control ($\chi^2_1 = 6.60$; $P < 0.05$) which suggests that some response to selection had been obtained.

A major problem was obtaining egg batches from fed females. To obtain single egg batches from females of the selection lines LOW 1 and LOW 2, they were isolated in filter paper lined tubes containing some water. Females of the FAJARA strain from which the lines originated were reluctant to lay in these tubes even when they appeared to be gravid. It was thought that this poor egg laying might have been due to low insemination rate.

When insemination rates in the FAJARA strain were measured, the results shown in Table 30 were obtained. As can be seen, the insemination rate for a density of 100 males/100 females only reaches 50% by day 8. Initially in the selection experiments, cages of about this density were used. At a later stage higher densities of mosquitoes were used where possible, and on some occasions artificial mating was attempted if the number of mosquitoes available was low.

4.3.4 Comparison between susceptibility to P. falciparum and P. v. nigeriensis

Infections of strains 4Y1, 156L1 (both refractory) and PYN1 (susceptible

Table 30. Insemination rates for A. gambiae s.s. FAJARA strain

Day no.	200/cage	400/cage
4	10%	44%
6	20%	58%
8	50%	76%

Table 31. Susceptibility to P. falciparum of A. gambiae s.s. strains 4Y1, 156L1 and PYN1 compared to the control FAJARA

Feed no.	N	% infected	G.M.(pos)	N	% infected	G.M.(pos)	χ^2_1
	C O N T R O L			4 Y 1			
5	11	100.0	122.7	9	100.0	62.1	
50	17	88.2	6.3	6	83.3	45.8	0.09,NS
53	50	46.0	8.6	7	100.0	22.0	5.18,*
56	30	73.3	10.0	50	90.0	31.3	2.70,NS
58	27	3.7	2.0	21	4.8	2.0	0.03,NS
	C O N T R O L			4 Y 1 (+)			
62	26	100.0	76.3	17	82.4	51.0	2.5[illegible],NS
	C O N T R O L			1 5 6 L 1			
8	13	15.4	2.5	5	20.0	4.0	0.0[illegible],NS
60	26	100.0	163.2	24	95.8	29.3	0.0[illegible],NS
62	26	100.0	76.3	25	56.0	3.9	12.[illegible],***
	C O N T R O L			P Y N 1			
10	16	31.3	3.0	62	24.2	2.5	0.1[illegible],NS

NS Not significant
* P < 0.05
*** P < 0.001

gambiae s.s. FAJARA strain

/cage	400/cage
0%	44%
0%	58%
0%	76%

parum of A. gambiae s.s. strains 4Y1, the control FAJARA

	N	% infected	G.M.(pos)	χ^2_1
		4 Y 1		
	9	100.0	62.1	
	6	83.3	45.8	0.09,NS
	7	100.0	22.0	5.18,*
	50	90.0	31.3	2.70,NS
	21	4.8	2.0	0.03,NS
		4 Y 1 (+)		
	17	82.4	51.0	2.59,NS
		1 5 6 L 1		
	5	20.0	4.0	0.06,NS
	24	95.8	29.3	0.002,NS
	25	56.0	3.9	12.10,***
		P Y N 1		
	62	24.2	2.5	0.13,NS

to P. y. nigeriensis) were carried out, concurrently with the FAJARA strain, by membrane feeding. The results of the feeds which produced at least some infected mosquitoes are shown in Table 31. In only two of the feeds (No. 53, involving 4Y1, and No. 62, involving 156L1) was there a significant difference between the percentage susceptibility of the experimental and control strains. In the other eight feeds, the susceptibilities of the two strains fed were found to be similar. This suggests that refractoriness to P. y. nigeriensis does not confer refractoriness to P. falciparum.

The strain 4Y1(+) in Table 31 is derived from 4Y1. After five months of colonisation in the MRC Laboratories, white-eyed mosquitoes were noticed in strain 4Y1. Since no other white-eyed colonies were kept there, contamination could not have been responsible. The w gene was found to be sex-linked, and by isolating single families of 4Y1, two strains were derived called 4Y1(+) (normal eyes) and 4Y1(w) (white-eyed). When 4Y1(w) mosquitoes were crossed reciprocally to the white-eyed strain SOK W after returning to London, all the offspring were white-eyed, This indicates that the w gene in 4Y1(w) is allelic with the w gene reported by Mason (1967). The 4Y1 kept in London had remained normal-eyed.

The FAJARA strain of A. gambiae s.s., which was found to be 100% susceptible to P. falciparum in The Gambia under the right conditions, was tested for its susceptibility to P. y. nigeriensis. Table 32 shows that it was only 37.3% susceptible to this parasite. This is further evidence that there is little relationship between the susceptibility of A. gambiae s.s. to the two different parasites.

Table 32 also shows that, with P. y. nigeriensis at least, there is no evidence that membrane feeding gives a lower estimate of infectivity (assessed by percentage infected) than direct feeds on a mouse. The G.M.(pos) was however lower from the membrane feed.

4.3.5 Frequency distribution of oocysts

Oocyst counts for 37 samples of various strains of A. gambiae s.s. fed on P. falciparum-infected blood were tested for goodness-of-fit to the negative binomial and Poisson distributions, using a computer programme written by Mr. T. Marshall (see Appendix 4). The programme

Table 32. Susceptibility of *A. gambiae* FAJARA strain to *P. y. nigeriensis* by mouse or membrane feed

	No. dissected	Percent infected	S.N.D.	G.M.(pos)
Mouse	51	37.3	0.018 N.S.	18.3
Membrane (20 mins)	16	37.5		3.0

Table 33. Results of χ^2 tests of goodness-of-fit to the negative binomial and Poisson distributions of oocyst counts for *P. falciparum*

	Number of samples differing at given P level						Total no. of samples
	N.S. (> 0.1)	< 0.1	< 0.05	< 0.01	< 0.005	< 0.001	
Negative binomial	32	4	1	0	0	0	37
Poisson	0	0	1	1	3	32	37

ae FAJARA strain to P. y. nigeriensis

cted	Percent infected	S.N.D.	G.M.(pos)
	37.3		18.3
		0.018 N.S.	
	37.5		3.0

dness-of-fit to the negative binomial
of oocyst counts for P. falciparum

differing at given P level				Total no. of samples
.05	<0.01	<0.005	<0.001	
1	0	0	0	37
1	1	3	32	37

calculates χ^2 for the difference between the observed and expected numbers of each count, and also calculates estimated values for the mean and the dispersion constant 'k' of the negative binomial. The numbers of samples which could be analysed was fairly restricted, since the computer programme grouped all counts over 99. If a large number of counts were over 99, the estimates of the mean and k were inaccurate and not meaningful in comparisons.

Table 33 shows the number of samples differing significantly from each of the distributions. In every case, the distribution of oocysts differed significantly from the Poisson distribution at the 5% level, but in only one case was it significantly different from the negative binomial at the same level. In that one case (Feed no. 65B) the mean number of oocysts per mosquito was low (2.88). The mean was also low in three of the four samples which differed significantly from the negative binomial at the 10% level (Feed nos. 10B, 41B, 50B and 64, which had means of 0.40, 0.29, 7.0 and 0.27 oocysts per mosquito respectively).

k, the dispersion constant of the negative binomial indicates the degree of 'overdispersion' or clumping in the distribution. The smaller the value of k, the stronger the overdispersion. A k value of about 8 is indicative of a non-overdispersed distribution (Southwood, 1978). Table 34 shows the values of the mean and k obtained for samples of the FAJARA strain of A. gambiae s.s. fed on P. falciparum-infected blood. It can be seen that k is in the range 0.05-1.86, i.e. the distribution of oocysts is strongly overdispersed.

Table 34. Parameters of the negative binomial distribution for oocyst counts of *P. falciparum* in *A. gambiae s.s.* FAJARA strain

Feed no.	Mean no. oocysts	S.E. (mean)	k	S.E. (k)
10A	0.88	0.52	0.23	0.15
22A	3.17	2.34	0.34	0.28
25	10.86	1.65	0.72	0.14
34A	14.85	7.60	0.10	0.03
35A	17.35	7.32	0.32	0.11
36A	28.77	5.19	0.65	0.13
B	35.30	3.76	1.86	0.38
C	66.04	9.21	1.40	0.31
41A	37.89	8.33	0.38	0.07
B*	0.29	0.24	0.05	0.04
43A	36.34	6.31	0.73	0.15
B	53.35	10.36	1.01	0.29
48A	14.42	4.35	0.22	0.05
49	6.35	1.15	1.26	0.37
50A	5.88	1.29	1.54	0.72
52A	23.18	4.80	0.63	0.14
B**	15.50	7.63	0.26	0.10
C	8.56	5.26	0.24	0.12
53A	8.18	2.99	0.15	0.04
56A	11.40	3.34	0.40	0.11
65A	49.40	8.15	0.88	0.18

* resuspended blood
** time intervals

tive binomial distribution for oocyst
m in A. gambiae s.s. FAJARA strain

S.E. (mean)	k	S.E. (k)
0.52	0.23	0.15
2.34	0.34	0.28
1.65	0.72	0.14
7.60	0.10	0.03
7.32	0.32	0.11
5.19	0.65	0.13
3.76	1.86	0.38
9.21	1.40	0.31
8.33	0.38	0.07
0.24	0.05	0.04
6.31	0.73	0.15
10.36	1.01	0.29
4.35	0.22	0.05
1.15	1.26	0.37
1.29	1.54	0.72
4.80	0.63	0.14
7.63	0.26	0.10
5.26	0.24	0.12
2.99	0.15	0.04
3.34	0.40	0.11
8.15	0.88	0.18

5. DISCUSSION

5.1 REFRACTORINESS TO P. v. nigeriensis

Observed susceptibilities of both the refractory and susceptible strains of A. gambiae s.s. fluctuated over the course of this work, even when no artificial selection was being carried out (Fig. 6). With a few exceptions, this was not due to variation in infectivity of mice, since control infections in A. stephensi were always high, with 100% of each batch infected in the great majority of experiments. Some authors (e.g. Rutledge et al (1970)) also observed changes in susceptibility of laboratory colonies, although in other cases, notably Al-Mashhadani et al (1980), susceptibility remained constant for several years.

In strains showing some heterozygosity for the gene(s) controlling susceptibility, it seems likely that the changes in susceptibility would be due to genetic drift which may occur when the population goes through 'bottlenecks' of small numbers. Alternatively, there may be selection for or against susceptibility, but it has been shown in this work that genes for refractoriness are not a cause of low fitness. A third explanation is that there was contamination between strains, which may be responsible for some of the large changes in susceptibility observed in Fig. 6.

Whilst the potential for disease transmission in a mosquito involves the ability to support both oocysts and sporozoites, assessment of susceptibility in the present work was based mainly on oocyst infections, to enable the intensity of infection to be determined by oocyst counts. If no oocysts form, there can be no sporozoites, and even one oocyst is unmistakable on a stomach dissected at least five days after the bloodmeal. In contrast to the work of Al-Mashhadani et al (1980), degenerate oocysts were never observed in mosquitoes of the refractory strains.

Inheritance of refractoriness to P. v. nigeriensis in A. gambiae s.s. was expected to be similar to that observed by Al-Mashhadani et al (1980) for P. berghei, since the strains used in the present work had most of their genome derived from the strains described by these

authors. They found susceptibility to be incompletely dominant, whilst in the present work the F_1 susceptibility was more exactly intermediate between the parental susceptibilities. It is notable that, as Al-Mashhadani et al (1980) had found, the F_1 from a cross with a male as refractory parent had significantly lower susceptibility than the reciprocal cross. The results of backcrosses do not suggest that either a sex-linked or an autosomal single gene controls refractoriness (Table 9). There is no evidence to contradict the finding of Al-Mashhadani et al (1980) that refractoriness to rodent malaria in A. gambiae s.s. is controlled by interacting polygenes.

5.2 FREQUENCY DISTRIBUTION OF OOCYSTS

It is obvious from any sample of infected moquitoes, whether P. y. nigeriensis or P. falciparum, that oocyst numbers are not normally distributed. This presents problems for the analysis of the intensity of infection and for making comparisons between the susceptibilities of different strains of mosquito.

The frequency distribution of oocysts is markedly right-skewed, with a high variance in relation to the mean. It was suggested some time ago (Eyles, 1951) that oocyst distributions might fit the negative binomial distribution (see Appendix 4) which is appropriate for many parasitic infections (Kennedy, 1975). Oocyst distributions in several mosquito/malaria combinations, including A. balabacensis and A. stephensi infected with P. falciparum, were found to fit the negative binomial but not the Poisson distribution (Rutledge et al, 1973). It seems therefore that oocysts are not randomly distributed with respect to each other, but are overdispersed (i.e. 'contagious' or clumped). This has been confirmed in the present work for P. falciparum in A. gambiae s.s. The values of k, the dispersion constant, obtained in the present work were in the same range as those of Rutledge et al (1973).

The intensity of infection in mosquitoes has been expressed in various ways in attempts to overcome the difficulties of analysing samples distributed according to the negative binomial. There is no straightforward way of transforming such data so that it is normally distributed, especially when k is variable between samples as it is in the case of

oocyst distributions. However, transformation to log(n + 1) gives a reasonable approximation to normality, at least when the number of zero observations is relatively small. The arithmetic mean or the median can also be used to describe the distribution, but with neither the geometric or arithmetic mean is it valid to perform statistical tests which depend on the data being normally distributed. In the present work, the geometric mean based on the transformation to log(n + 1) was used, partly for expediency because in samples with high oocyst counts (as obtained in control and susceptible strains with P. y. nigeriensis) the high counts could be estimated instead of counted accurately, with little effect on the geometric mean.

What explanation is there for the non-random distribution of oocysts in mosquito infections? Differences in infectibility between mosquitoes could arise because each mosquito takes up a non-random number of gametocytes. Kennedy (1975) suggested that one of the most important reasons for differences in infectibility is likely to be genetic variation in susceptibility of the mosquito or infectivity of the parasite. However, Rutledge et al (1973) showed that genetically uniform, selected lines of Ae. aegypti infected with a strain of P. gallinaceum still had oocyst distributions conforming to the negative binomial. This finding suggests that genetic variability of host and parasite is not responsible for the non-random nature of the oocyst distribution.

It should be remembered that, during gametogenesis in the mosquito midgut, each microgametocyte of P. falciparum gives rise to eight microgametes. The sex ratio amongst gametocytes is usually heavily biased in favour of females (see Table 23). Therefore, as Rutledge et al (1973) pointed out, if conditions in the midgut combine to be favourable for infection, there will be an 'amplification' process, whereby chance differences in the number of microgametocytes ingested will be magnified. This seems likely to make a large contribution to the non-random distribution of oocysts.

5.3 INFECTIVITY OF P. falciparum-INFECTED BLOOD

For maximum infections to be obtained by the present membrane feeding technique, it was found that mosquito feeding should be completed within 15 minutes or less of the blood leaving the patient. This result is similar to those of Collins et al (1964) with three other

anophelines. However Yoeli (1938) found that defibrinated P. falciparum-infected blood remained infective to A. elutus after five hours at 23-27°C or 72 hours on ice, whilst Dashkova and Rasnitsyn (1978) obtained high infections in A. atroparvus after holding P. vivax-infected blood for one and a half to two hours. The different results of these authors may be due to the use of anticoagulants other than heparin.

Understanding of the normal process of gametogenesis may shed light on the reasons for loss of infectivity of gametocytes. Once blood leaves a gametocyte carrier, gametogenesis is remarkably rapid, with exflagellation occurring at about 9 minutes in P. falciparum (Sinden et al, 1978). It might seem, therefore, that the timing of exflagellation is consistent with the short time available for optimally infective feeding in a membrane feeder. It was found, though, that maintaining the blood at 37°C in a membrane feeder prevented the majority of gametocytes from activating. The effect of temperature in control of gametogenesis in vitro has previously been demonstrated in P. y. nigeriensis and P. falciparum (Sinden & Croll, 1975; Sinden & Smalley, 1976). It appears to act instead of, or in addition to, the effects of pH and 'mosquito factors' shown to operate in P. gallinaceum (Carter & Nijhout, 1977; Nijhout, 1979).

The prevention of gametocyte activation did not appear to preserve gametocyte infectivity however. It is not known whether gametocytes which become activated or fertilized in the feeders are subsequently able to infect mosquitoes. However, even if they are not, the rate of loss of gametocytes by activation would not be sufficient to explain the rate of decline of infectivity to mosquitoes. It is possible that holding the blood at 37°C in the feeders causes irreversible inactivation of gametocytes. However the rise in the observed percentage activated during the period 35 to 55 minutes after bleeding suggests that at least a proportion of those not activated during the feeding period retained the capacity to activate (Table 23). Irreversible inactivation of gametocytes could be due to exhaustion of energy-supply, although it seems unlikely that this would be the case during such a short time in the membrane feeders, given that gametogenesis is largely inhibited. The most likely explanations for loss of gametocyte infectivity are either mechanical damage to gametocytes during their time in the stirred feeders, or the effect of heparin.

Apart from its uses in the study of mosquito susceptibility, membrane feeding is becoming increasingly important as methods progress for the *in vitro* culturing of *Plasmodium* with the production of gametocytes (Smalley, 1976) and gametogenesis (Carter & Beach, 1977). When used to infect mosquitoes for the mass production of sporozoites (e.g. Nardin *et al*, 1979), an extended feeding time of 25-35 minutes is more suitable; but for these longer feeding times a stirrer is essential to prevent settling of the red cells, as was shown by Wade (1976). Settling of cells, which is particularly rapid in high gametocyte carriers who tend to be anaemic, leads to mosquitoes obtaining only serum feeds.

In the present work little emphasis was placed on establishing the lowest gametocytaemia which would yield any infection, since interest was centred on selecting those patients whose blood would give high mosquito infections. The lowest gametocytaemia which produced an infection was 56/mm^3, but a lower limit of 300 gametocytes/mm^3 was found to be useful for selecting donors.

The membrane feeding technique was found to simulate quite well the natural feeding process, as can be seen by comparing the results with those obtained by direct feeding. Bray *et al* (1976) conducted 30 feeds in The Gambia on 12 donors with 267-15,520 gametocytes/mm^3. In 27 of the feeds the patients had more than 300 gametocytes/mm^3 but in only 13 of these were 70% or more of batches of mosquitoes infected, as compared to 21 out of 31 membrane feeds in the present work on blood samples with more than 300 gametocytes/mm^3 which infected at least 70% of mosquitoes. However, direct feeds on young children gave a high proportion of partial feeds (Bray, personal communication).

Since the history of infection in gametocyte donors was not known, some of the factors affecting the infectivity of gametocytes cannot be assessed. Nevertheless it is clear from Fig. 21 and Table 27 that infectivity to mosquitoes increases with gametocytaemia. There was no evidence that the proportion of infective carriers was different in each age-group. This fact supports in general the suggestion of Smalley and Sinden (1977) and Carter *et al* (1980) that infectivity to mosquitoes is not affected by immunity to gametocytes. However, the observation of phagocytosis of intracellular gametocytes by Sinden *et al* (1978) suggests that some individuals at least may be able to

produce transmission-blocking immunity. This may be the explanation for the five people out of a total of 24 in the present work who had more than 300 gametocytes/mm^3 but who were completely non-infective to mosquitoes.

5.4 REFRACTORINESS TO P. falciparum

Selection of a strain of A. gambiae s.s. completely refractory to P. falciparum was not successful, although in one line a decreased level of susceptibility was achieved after three generations of selection. The poor response to selection could indicate that genes for refractoriness are rare or absent from the FAJARA strain, and this was suggested by Fig. 22 which showed that at high average infection intensities, 100% of the mosquitoes were infected. This is in contrast to the case reported by Eyles & Young (1950), who showed that the percentage of a batch of A. quadrimaculatus infected with a strain of P. falciparum never rose above 90%, even when the mean number of oocysts per gut was over 100. A. albimanus similarly had a maximum infection rate of 70%. With a different strain of P. falciparum, the situation was reversed; whilst A. albimanus was 100% susceptible, batches of A. quadrimaculatus were only about 80% susceptible at high infection intensity (Jeffery et al, 1950). These results suggest that selection for refractoriness on particular strains of A. quadrimaculatus or A. albimanus might be fruitful.

The lack of response to selection in A. gambiae s.s. may, however, have been for technical reasons. At the start of the work with P. falciparum, the time allowed for membrane feeding was too long, and a number of females selected because they were not infected were presumably not refractory. Other difficulties included the problems of persuading small numbers (i.e. single families) of A. gambiae s.s. to feed on membranes, the unpredictability of availability of gametocytes at each generation, and the non-infectivity of some high gametocyte carriers. For these reasons it would be wrong to conclude from the present work that the prospects for selecting a P. falciparum - refractory strain of A. gambiae s.s. are hopeless, but it should be recognised that such a project would be very laborious and time-consuming.

Despite these problems, it was possible to investigate the susceptibility to P. falciparum of the strains of A. gambiae s.s. which are refractory or susceptible to P. y. nigeriensis, and show that there was no difference between their susceptibilities and that of the FAJARA

control. This result is in contrast to that of Al-Mashhadani and Davidson (1976) who had reported not only that the P. berghei-refractory line of A. gambiae s.s. was refractory to two other rodent malarias but also that in two out of four feeds on P. falciparum-infected donors, the refractory line showed decreased susceptibility to this parasite. The present discouraging result that there is no cross-refractoriness between rodent malaria and P. falciparum means that refractoriness to human malaria can only be studied with the parasite in question, obtained either from humans, primates or preferably cultures.

5.5 INTRODUCTION OF REFRACTORINESS GENES INTO POPULATIONS

In the A. gambiae complex, the only means available at present for the introduction of desirable genes into wild populations is 'dilution'. Although this might seem to be an inefficient method compared with methods which actually 'drive' a desirable gene, in fact it has certain advantages. Since there is no sterility in cross-matings between the released and wild types, the desirable gene(s) would be able to recombine freely into a background of field-adapted genes. The released strain must have sufficient fitness to achieve a proportion of matings in the wild, but otherwise need not be as well-adapted to field conditions as would be required by replacement methods such as cytoplasmic incompatibility or compound chromosomes. Lack of sterility in cross-matings also means that there would be no selection pressure favouring females who discriminate against released males. However, the disadvantage of 'dilution' is that the replacement process does not accelerate as releases progress (as it would in methods involving negative heterosis).

The advantages of the 'dilution' method are well illustrated by the cage replacement experiment. The refractory strain 4Y1, which was used for releases, showed very poor performance in the laboratory, with a low egg yield per female and low egg hatchability. Although its mating competitiveness for PYN1 females was only 25% compared with PYN1 males, releases were sufficiently numerous and prolonged to reduce the susceptibility to P. y. nigeriensis in the cage population considerably. The level of susceptibility remained low for many months after releases had ended, and it is considered that this encouraging result could only have been achieved as a result of recombination separating refractoriness gene(s) from those causing low

fitness.

It was also shown theoretically that the hybrid sterility in the A. gambiae complex could be used as a powerful method of introducing desirable genes. Since female hybrids in the A. gambiae complex are fertile, hybrid sterility could not be used to replace one complete genome by another, and it is therefore similar to 'dilution' in that the 'desirable' part of the genome could recombine with other wild-type genes. The 'desirable' part considered in the calculations was the X-chromosome, because of the demonstration by Al-Mashhadani et al (1980) that the X-chromosome of A. gambiae s.s. carries a gene affecting susceptibility to P. berghei.

The replacement of one X-chromosome by another would operate by negative heterosis, since each sterile hybrid eliminates one gambiae s.s. and one arabiensis sex chromosome and this represents a larger proportion of whichever is in the minority. Establishment of a majority of the sex chromosomes of one species (by releases) would therefore inevitably lead to elimination of the other.

In order that the released males and females have good mating competitiveness with the wild-type, release of strains with the X (carrying refractoriness) and Y-chromosomes of one species, in the background of autosomes partly derived from the 'target' species, are envisaged. These types of strains have been produced with the X and Y-chromosomes of either gambiae s.s. or arabiensis (Curtis, 1978b,c). If the Y-chromosome were involved in mating behaviour, as was suggested by Fraccaro et al (1977) in the A. maculipennis complex, then males of such synthesised strains would not be competitive and hybrid sterility could not be used to introduce a desirable X-chromosome.

When considering whether the introduction of refractoriness genes would actually cause the level of malaria transmission to be reduced, we need to assess the effect such genes would have on the vectorial capacity of a mosquito population. Vectorial capacity is the average number of innoculations, originating from one case of malaria in unit time, that a vector population would distribute to man if all the vector females pick up the infection (Garrett-Jones, 1964). In other words, it is the number of potentially infective contacts a person

makes, through the vector population, in unit time. The vectorial capacity depends on the mosquito density, man-biting habit, probability of daily survival and the duration of the extrinsic cycle of the parasite in question. Vectorial capacity is not the same as the basic reproduction rate, i.e. the number of secondary cases generated from one infectious case (Macdonald, 1957). There is a 'critical level' of vectorial capacity below which the basic reproduction rate is less than one, i.e. below which malaria cannot maintain itself at an endemic level.

Whilst mosquito susceptibility is not usually considered as a factor in vectorial capacity (except in so far as it is assumed to be 100%), there is no doubt that refractoriness in the mosquito population would reduce the vectorial capacity in direct proportion to the percentage of phenotypically refractory mosquitoes. The amount of reduction required to bring the vectorial capacity down to the 'critical level' obviously depends on its original level. In one village studied in the Garki district, Nigeria, it was calculated that the vectorial capacity of a population of A. gambiae s.l. would have had to be reduced by a factor of more than 170 in order to reduce the yearly average parasite rate to half its original value (Dietz et al, 1974).

It is clear that such drastic reductions in vectorial capacity present problems for other methods of control (e.g. insecticides) as well as for replacement by refractory types. The 'insecticidal impact' on A. gambiae s.l. vectorial capacity was rarely found to be high enough to prevent transmission (Garrett-Jones & Grab, 1964). In one example, DDT spraying of a population of A. gambiae s.l. reduced the vectorial capacity by a factor of 23, whilst the reproduction rate of malaria remained at about 20 (Garrett-Jones & Shidrawi, 1969).

However, even the estimates of insecticidal impact measured by the methods of Garrett-Jones and Grab (1964) may be misleadingly high, since it is known that members on the A. gambiae complex show behavioural divergences in their resting behaviour (Coluzzi et al, 1977). The assumption that there were two sub-populations (one exophilic and one endophilic) in a population of A. gambiae s.l. led to a smaller but more realistic estimate of the insecticidal impact on vectorial capacity (Molineaux et al, 1979). It can be said in favour of the introduction of refractoriness genes that there would be no

selection pressure favouring evasive behaviour, or indeed favouring straightforward insecticide resistance. It may in fact be selectively advantageous to a mosquito to be refractory.

5.6 FITNESS OF REFRACTORY TYPES

When considering the effect of fitness on the likelihood of a refractory type remaining in a population once introduced, there are three factors to be taken into account. The first is whether refractoriness confers low fitness on a mosquito in the absence of infection. The second is whether the parasite causes damage to its host, and the third is whether refractoriness improves the survival of mosquitoes exposed to infection. The third factor may be able to outweigh the effects of the first, but it is clear that refractoriness may only be selectively advantageous to a host in a situation where the parasite causes damage such that the reproductive success of at least a proportion of the hosts is reduced.

We therefore have to consider the effects of host refractoriness genes both in the absence and the presence of parasites. Cage population studies with mixed genotypes of mosquito maintained on uninfected blood showed that there was no strong selection against either the f^m or the pls locus in Ae. aegypti (Macdonald, 1965; Kilama, 1973). Thus it appears that in the case of Ae. aegypti, refractoriness to either P. gallinaceum or to filaria has no deleterious effect in the absence of infection.

In the present work, it seemed at first that for A. gambiae s.s. and P. v. nigeriensis the opposite might be true, since the two refractory strains 4Y1 and 156L1 were difficult to rear and compared poorly with the susceptible strain in many respects. However, the results from the cage replacement experiment, which was maintained on uninfected blood and in which the proportion of refractory types remained constant for eight months (approximately 15 generations) without further releases or artificial selection, indicated that refractoriness did not necessarily confer low fitness on a mosquito, since otherwise susceptibility would no doubt have increased in frequency over such a period of time. The low fitness of the 4Y1 and 156L1 strains must be due to other factors and may be explained by inbreeding depression.

The numerous reports of effects of parasites, including filariae and malaria, on their insect vectors have been summarised by Maier (1976). The damaging effect of microfilariae is well known. In susceptible Ae. aegypti, mortality after B. pahangi infection occurs in two distinct phases, at the times when filarial larvae are migrating to the thorax and into the haemocoel respectively (Townson, 1971). Survival of refractory mosquitoes was as good as uninfected controls during the later period of infection, whilst during the early period of mortality heterozygotes at the f^m locus survived better than either refractory or susceptible homozygotes (Townson, 1971). When Macdonald (1965) set up population cages with different initial frequencies of f^m and maintained them on infected blood, the equilibrium frequency of f^m was found to be higher than in the control uninfected cages. This could have been due to heterozygous advantage maintaining the gene frequency of f^m despite its deleterious effects when homozygous in the presence of filaria; an alternative explanation is that the level of microfilaraemia used to infect the populations was too low to cause any decreased survival in any case.

Malaria infection in mosquitoes can reduce fecundity (Freier & Friedmann, 1976), flight ability (Schieffer et al, 1977) and survival (Gad et al. 1979a). Decreased survival of A. gambiae s.s. after a P. v. nigeriensis-infected bloodmeal was noted by Prasittisuk (1979). Infection with P. v. nigeriensis in A. gambiae s.s. and A. stephensi was also found in the present work to lead to increased mortality, particularly in the first two days after the bloodmeal, compared to uninfected batches. The most interesting feature of the results is that the refractory and susceptible types both suffer from increased mortality. Fecundity of infected refractory and susceptible strains was not investigated in the present work. In the case of P. gallinaceum in Ae. aegypti, the fecundity of a non-susceptible strain was less severely reduced after an infected bloodmeal than a susceptible strain, whilst hybrids had fecundity which was higher than either parent strain (Hacker, 1971).

There is therefore very little evidence about whether refractoriness to malaria parasites gives a mosquito selective advantage when exposed to infection, despite the fact that a deleterious effect of infection has been demonstrated in many cases including the present work. It should again be remembered, though, that A. gambiae s.s.

is not a natural vector of rodent malaria. A damaging effect of human malaria on mosquitoes has yet to be demonstrated. Nevertheless, it seems possible that in genetic control projects malaria-refractoriness genes would get a 'helping-hand' from their superior fitness in the presence of infection.

If fully refractory mosquitoes did have a selective advantage because of decreased mortality when infected, then it would be expected that wild populations of mosquito would have refractoriness genes at a high frequency. In the one case in which the geographical distribution of genes for refractoriness to malaria has been investigated, Kilama (1973) found that the gene pls (for refractoriness to P. gallinaceum) was not present in five strains of Ae. aegypti from Asia or the Americas, but was present in all eight strains tested from Africa. The gene frequency of pls ranged from 0.27-1.0 in those strains which carried it.

However, it is not necessarily expected that a refractoriness gene would be at a frequency of 100% in wild populations, since we have to take into account the ability of the parasite to evolve in parallel with the mosquito host. 'Gene-for-gene' relationships of resistance and virulence are well known in plant pathology. Examples of virulence genes in malaria parasites for their mammalian hosts are well known (Walliker et al, 1976), and there seems to be no reason why similar genes could not exert their effects in the insect host. The observations of close adaptation of malaria parasites to their local vectors (e.g. Eyles & Young, 1950; Jeffery et al, 1950) may be examples of this effect.

Heterozygous advantage, in the form of generalised increase in resistance to parasites of all virulence types, may be responsible for maintaining a polymorphism of refractoriness/susceptibility genes in a mosquito population. However Clarke (1976) has shown that the host-parasite interaction could also maintain a polymorphism as a 'stable limit cycle', which means that there are cyclically fluctuating frequencies of 'resistance' and 'virulence' genes rather than a stable equilibrium frequency. Such a cycle will only arise if a resistance gene has a selective advantage when the host is infected by one particular parasite type, but a disadvantage when it is infected by the other. As the frequency of a resistance gene increases, its selective advantage decreases as natural selection favours variant parasites

which can attack the resistant host, but which have reduced ability to attack the old susceptible type. A feedback loop may be set up as the majority host genotype then reverts back to the original type. There is not yet enough evidence of the increased fitness of mosquito refractory types in the presence of malaria parasites to suggest that such cycles might be occurring naturally.

5.7 GENERAL CONCLUSIONS

In the laboratory it is possible to introduce refractoriness genes into a population of *A. gambiae s.s.* by 'dilution'. Given enough time, it is likely that the level of susceptibility could be reduced to a level as low as that of the refractory strain. Refractoriness genes do not confer low fitness on a mosquito since the level of susceptibility stayed low once the releases had ended.

The replacement experiment illustrated that the introduction of refractoriness genes by 'dilution' does not require refractoriness to be controlled by a single gene, since in the refractory strains used it was found to be controlled by interacting polygenes. This finding rules out for the present the possibility of using hybrid sterility as a means of introducing refractoriness genes.

To decrease the vectorial capacity of a wild population of *A. gambiae s.s.*, genes for refractoriness to human malaria are required. These appeared to be absent or at a very low frequency in the FAJARA strain, and genes for refractoriness to *P. y. nigeriensis* were not effective against *P. falciparum*.

The difficulties of working with *P. falciparum* from naturally acquired infections by membrane feeding mean that selection of a *P. falciparum*-refractory strain would be extremely time-consuming. The prospects for population replacement by a *P. falciparum* refractory type are therefore more remote than was previously hoped.

The study of refractoriness to *P. y. nigeriensis* and *P. falciparum* in *A. gambiae s.s.* raises interesting questions about the adaptation of mosquito/malaria systems, the distribution of oocysts in mosquitoes, and the infectivity of gametocyte carriers which deserve to be further investigated.

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APPENDIX 1 : Nomenclature

A : The Anopheles gambiae complex

There are six known species in the A. gambiae complex (Davidson et al, 1967; Davidson & Hunt, 1973). The freshwater forms were formerly called species A, B, C and D. Recently White (1975) and Mattingly (1977) have adopted the following names which are used in the present work: A. gambiae Giles (1902) for species A; A. arabiensis Patton (1905) for species B; A. quadriannulatus Theobald (1911) for species C. Species D is as yet unnamed. The saltwater species retain their original names of A. melas Theobald (1903) and A. merus Dönitz (1902).

A. gambiae (formerly species A) is here called A. gambiae s.s. (sensu stricto), whilst an unspecified member of the complex is called A. gambiae s.l. (sensu lato).

B : The Culex pipiens complex

The members of this complex which are mentioned in the present work are as follows.

- C. pipiens L (1758): This name is used by some authors to refer only to the non-autogenous, non-man-biting form (C. p. pipiens), whilst others (mainly American authors) include the urban autogenous form (C. p. molestus Forskål (1775)), under the name C. pipiens. Where the 'subspecies' is known, it has been specified; otherwise C. pipiens may be taken to mean either C. p. pipiens or C. p. molestus.
- C. quinquefasciatus Say (1823): the species which was previously referred to by all but American authors as C. p. fatigans Wiedemann (1828). C. quinquefasciatus is now accepted as the correct name (Sirivankarn & White, 1978). This species is the widely distributed vector of human filariasis.

C : Rodent malaria parasites

Parasites of the P. berghei group are regarded as belonging to the two species berghei and yoelii. Following Killick-Kendrick (1974), these names are used:

- P. berghei Vincke and Lips (1948) (formerly P. b. berghei).
- P. yoelii nigeriensis Killick-Kendrick (1973) (formerly P. b. nigeriensis).

APPENDIX 2 : Percentage infected with P. y. nigeriensis in samples from the cage replacement experiment, and concurrent control infections

Week no	Exp't no	Releases	CAGE POPULATION No dissected	CAGE POPULATION % infected	'BEECH' CONTROL No dissected	'BEECH' CONTROL % infected
6	21		75	100.0	16	100.0
7	23		21	100.0	24	95.8
13	29		11	100.0	8	100.0
18	31		24	100.0	7	100.0
20	33 A/B		50	96.0	22	100.0
22	34		57	93.0	41	100.0
24	36A		13	92.3	5	100.0
26	38		15	100.0	6	100.0
28.5	41		11	90.9	8	100.0
30	42	4Y1	13	84.6	6	100.0
32	43		62	93.5	42	100.0
34	44		26	65.4	4	100.0
36	45		26	76.9	44	100.0
37.5	48		12	41.7	5	80.0
38	49		48	70.8	19	94.7
73	50		47	38.3	8	100.0
77.5	52		50	48.0	28	100.0
78	55		15	40.0	All died before dissection	
80	58	4Y1	50	68.0	18	100.0
82	60		50	54.0	6	100.0
84	62		6	33.3	2	100.0
86	65		29	58.6	17	100.0
90	68		41	41.5	5	100.0
92	70		50	66.0	25	96.0
95	74		4	50.0	6	100.0
100	76		7	28.6	7	100.0
102	77		18	27.8	29	100.0
104	80	15&14.2	38	57.9	10	100.0
106	81		35	54.3	8	100.0
108	82		32	31.3	24	100.0
110	83		80	30.0	5	100.0

APPENDIX 3 : Intensity of infection with P. y. nigeriensis in samples from the cage replacement experiments, with concurrent control infections

Week no	Exp't no	Releases	CAGE POPULATION GM	CAGE POPULATION GM(pos)	'BEECH' CONTROL GM	'BEECH' CONTROL GM(pos)	Standardised GM(pos)
6	21		90.9	96.6	120.2		80.4
7	23		245.7		70.0	84.1	292.2
13	29		154.6		192.6		80.3
18	31		90.1	109.6	144.7		75.7
20	33A		41.9	60.9	251.0		24.3
	33B		63.7	79.7	42.8		186.2
22	34		55.7	75.4	198.3		38.0
24	36A		22.1	87.2	71.4		122.1
26	38		110.1		251.0		43.9
28.5	41		91.6	143.9	229.1		62.8
30	42	4Y1	37.8	73.2	251.0		29.2
32	43		107.9	149.1	143.9		103.6
34	44		6.8	22.7	251.0		9.0
36	45		20.6	51.2	166.1		30.8
37.5	46		4.2	30.5	50.1	133.4	22.9
38	49		17.8	58.3	81.5	104.1	56.0
73	50		4.1	39.7	251.0		15.8
77.5	52		3.6	14.2	35.2		40.3
78	55		7.1	136.2	(all died, assume 251)		54.3
80	58	4Y1	20.6	85.8	251.0		34.2
82	60		8.0	47.1	251.0		18.8
84	62		3.3	36.0	251.0		14.3
86	65		7.7	32.8	245.8		13.3
90	68		3.6	22.7	251.0		9.0
92	70		14.5	57.7	201.2	251.0	22.9
95	74		7.1	50.0	251.0		19.9
100	76		3.9	116.4	251.0		46.4
102	77	15614.2	3.6	97.4	243.4		40.0
104	80		10.3	56.5	251.0		22.5
106	81		14.9	144.6	251.0		57.6
108	82		3.3	43.9	251.0		17.5
110	83		2.0	10.3	61.3		16.9

APPENDIX 4 : The negative binomial distribution

This distribution is described by two parameters, the mean and the exponent k, which is a measure of the amount of overdispersion or clumping (sometimes called contagion). The probability of obtaining a particular value, r, is given by the formula (from Southwood, 1978, p. 30):

$$P_r = \frac{\Gamma(k+r)}{r!\,\Gamma(k)} \times \left(\frac{\mu}{\mu+k}\right)^r \times \left(\frac{k}{k+\mu}\right)^k$$

where μ = mean
k = dispersion constant

A computer programme made available by Mr. T. Marshall was used to test whether a sample of oocyst counts conformed to the negative binomial. The programme first estimated k from the mean and variance, since (Southwood, 1978, p. 28):

$$\hat{k} = \frac{\bar{x}^2}{s^2 - \bar{x}}$$

where $\bar{x}$ = estimate of mean
s^2 = variance

The value of k was found by repeatedly calculating new estimates of k which gradually converged on the maximum likelihood value. Once k was estimated, the probability for each count could be calculated from the first formula above. In practice, the programme calculated probabilities for all counts up to 99, and pooled the remaining probabilities.

χ^2 was evaluated from the observed and expected frequencies of each count. The degrees of freedom were 3 fewer than the number of classes of counts. In many of the samples, the spread of counts was high compared to the sample size, and consequently the expected value for each count was low. For these samples, the number of degrees of freedom may have been artifically high. Since in most cases, the χ^2 value was well below that of the 5% significance level, the elevated number of degrees of freedom was not important in changing the interpretation of the results.

The programme also calculated the expected frequencies for each count from the Poisson distribution, where the probability of a value r is:

$$P_r = \frac{\mu^r e^{-\mu}}{r!}$$

where μ = mean

The χ^2 from this comparison of observed and expected counts had 2 fewer degrees of freedom than the number of classes of counts.